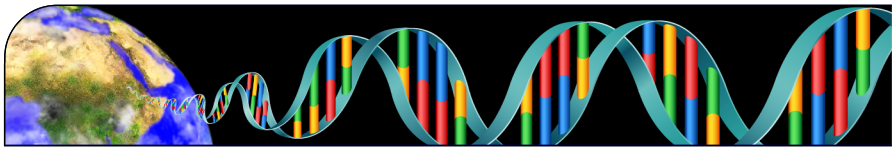


REDISCOVERING BIOLOGY

Molecular to Global Perspectives

Overview and Credits

Introduction to Project

Goal and intended audience

Rediscovering Biology was designed for high school biology teachers who have substantial knowledge of basic biology but who want to learn about important new discoveries of the last two decades. It was also designed so teachers could familiarize themselves with research methods and tools that will lead to new discoveries in the coming decade. In designing the project we asked: What do teachers already know? What information would help them better understand recent and future developments?

This is not a curriculum development project and does not attempt to provide materials for use in the high school classroom. In most cases, the level of presentation is too advanced for those who are beginning the study of biology. But through exposure to the research methods and techniques used by today's scientists, and with an understanding of some important new concepts, we hope that teachers will gain a heightened appreciation of ideas they already teach, as well as an increased ability to incorporate new topics into their curriculum.

Other users — such as college students, advanced high school students, professional scientists, graduate students from other fields, or well-educated laypersons — may also find this project useful. We welcome their use of these materials.

The materials were designed to be used in various ways. Some individuals may want to learn about a single topic and study parts of one unit on their own. Some may join in small facilitator-led groups, such as professional development in-service sessions, to go over one or a group of related units. Others may choose to complete the entire course. For the latter group, graduate credit may be earned through Colorado State University. For information on earning credit or obtaining materials go to:

http://www.learner.org/channel/workshops/graduate_credit.html.

How topics were chosen

The teachers and researchers on our advisory board each proposed ten to twenty areas of biology that they thought had undergone significant change in the preceding decade. The cumulative list was then combined and narrowed down to thirteen major unit topics that the group agreed would provide a good foundation for those wanting to learn about new developments in biology.

This is not a comprehensive treatment of the field of biology. It includes areas of study that may be entirely new to some, such as genomics and proteomics. It also includes more traditional topics, such as human evolution and neurobiology, which have changed substantially because of the application of new techniques. Indeed, a common theme throughout the project is the application of processes and techniques at the molecular level to enlighten studies of organisms, populations, or ecosystems.

Assumptions about user knowledge

We assume that users of this material have knowledge equivalent to that of someone with a bachelor's degree in a biological science. Most terms and concepts that are used in a high school biology text are not defined or explained. We also recognize that biology is a rapidly advancing field, and someone who graduated from college a decade ago could not have been exposed to some of what is taught today. Biology is also a huge field of study, and many students who graduate from college this year, even, will not have been exposed to all of the material collected here. Many of the concepts we explore can be found in a recent introductory college biology textbook such as Freeman's *Biological Science*, or Campbell and Reece's *Biology*. Users might find it useful to have access to such a text as an additional reference.

Project Components

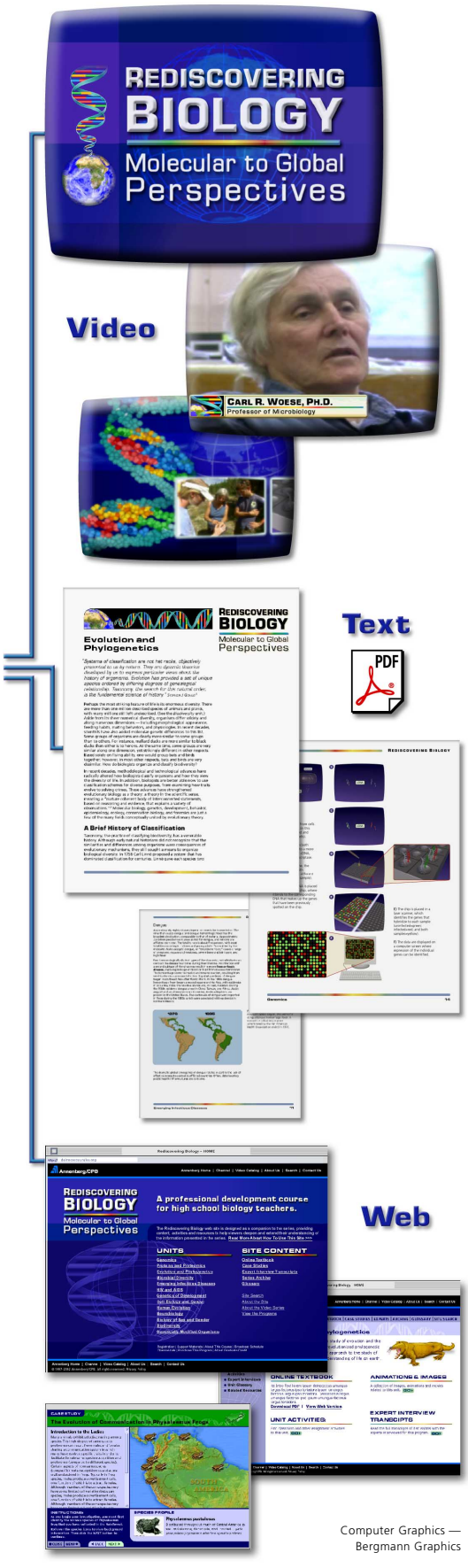
Rediscovering Biology is a multimedia project. Each of the thirteen units comprises a half-hour video, an online text chapter, and a set of learning activities. The Web site provides access to all of these, as well as additional resources, including:

- a glossary that serves as a navigational tool to other parts of the project
- interactive case studies
- transcripts from expert scientist interviews
- animations from the videos and case studies
- still images from the videos and text book

The videos and the text chapters can be used independently; if both are used, it is possible to start with either one. We imagine that most users will watch the video first, then read the chapter, and then perhaps watch the video again.

Each video includes interviews with two or more expert scientists. Through these interviews viewers will get a sense of how and why these scientists do their research, and will have a look at some of the equipment and techniques they use. In choosing experts to interview, we looked for those who are nationally and internationally recognized, regardless of their gender or ethnicity. Should you wish to know more about the work of a particular researcher featured in the videos, the full transcripts from the interviews with these experts are available on the Web site.

The Web site is both to organize the different components of the project and a place to go for additional information. On the Web, a comprehensive glossary defines important terms used throughout the series, and provides links to text and animations where these concepts are explained or used. Animations from the videos are available on the Web site so that users may study them in more detail, playing them



Computer Graphics — Bergmann Graphics

repeatedly or pausing in the middle to study them. Transcripts of interviews of scientists provide a rare opportunity to get to know scientists who are associated with many of the leading discoveries in biology today and understand their research.

Order of units

Users may decide to study all thirteen units or they may be interested in a single one. Each unit is meant to stand alone, but we often refer to ideas and techniques presented in other units. We have organized the units so that techniques such as microarray analysis or BLAST searches, which are used in several units, are explained early in the series. An html form of the text is available on the Web; from it users may navigate through the various units and the different components.

Online text

The online text chapters are not simply a repeat of what is in the video. Rather, they show how information from the video fits into the larger field. In other words, they provide context for the focused examples presented in the video. One central theme present in nearly all of the chapters of the online text is the role that genetics and genomic studies have had in increasing our understanding of the various fields of biology.

Each chapter was written by one of three authors, selected for his or her knowledge of biology and ability to write clearly about that knowledge. All of these authors have taught at the college level. The chapters vary somewhat in style and level of difficulty; these differences result both from the nature of the material itself, as well as from differences among writers.

Authors

Amy Does, PhD, is a microbiology instructor at Portland Community College in Portland, Oregon. In addition to teaching prenursing students, she provides professional development for elementary school teachers who conduct afterschool science clubs. She has developed exhibits for a science museum, designed science software for middle school students, and taught college-level biology online. Amy is the author of the Microbial Diversity, Emerging Infectious Diseases, HIV and AIDS, and Genetically Modified Organisms chapters.

Norman A. Johnson, PhD, is an adjunct research assistant professor at the University of Massachusetts at Amherst. His research has focused on speciation and several other areas of evolutionary genetics. In addition to the University of Massachusetts, Norman has also taught at the University of Chicago and the University of Texas at Arlington. Norman served as the style editor for all thirteen chapters, and is the author of the Evolution and Phylogenetics, Genetics of Development, Human Evolution, Neurobiology, and Biodiversity chapters.

Teresa Thiel, PhD, is a professor of biology at the University of Missouri-St. Louis. Her main interests are molecular biology, microbiology, and bioinformatics. She directs a program for high school teachers and students called “Science in the Real World: Microbes in Action” that includes a Web site of the same name. She teaches microbiology and microbial genetics to undergraduate and graduate students, and offers summer workshops in microbiology for teachers.

Teresa is the author of the Genomics, Proteins and Proteomics, Cell Biology and Cancer, and Biology of Sex and Gender chapters.

Learning activities

Each unit contains several learning activities tailored to the information in the unit. These activities include simple review and discussion questions; exercises that demonstrate how data are generated, interpreted, and applied; explorations of ethical issues; and consideration of how the information relates to other fields. Most of the activities assume the participants are familiar with the unit's video and online text.

Case studies

Four interactive Web-based case studies showcase a specific area of applied or basic research in cancer, comparative evolution, HIV, or genetically modified organisms. Each case study takes the participant through a series of steps in a research project. After viewing explanatory and background material on the project, the participant chooses an experiment to perform or a hypothesis to test. The case studies provide an interactive experience that complements the video and text chapters; and provide a window into the choices, challenges, compromises, and rewards associated with one area of biological inquiry. Each case study is an independent activity but may incorporate information from more than one unit.

Because the case studies go into greater depth than the videos and texts, and rely on information from them, it is best to do them after completing the other components. The first Web page of each case study provides links to the videos and online texts that are relevant to the study.

Writers

Chris Tachibana, PhD, has taught undergraduate biology since 1992 at Salt Lake Community College, Penn State University, and the University of Washington. She is a research scientist at the University of Washington Biochemistry Department and the Carlsberg Research Labs in Denmark. Chris developed two case studies: The Genetics of Resistance to HIV and Designing an Anti-Cancer Drug. She also authored the learning activities for the Genomics, Proteins and Proteomics, Emerging Infectious Diseases, HIV and AIDS, Cell Biology and Cancer, Biology of Sex and Gender, and Genetically Modified Organisms units. In addition, she produced the learning activity course guides for all thirteen units, and gave the learning activites for all units a common voice.

Andrea (Andi) White, PhD, is a postdoctoral research associate at the University of California, Berkeley. As a graduate student at the University of New Hampshire she was a teaching assistant for marine ecology, honors biology, economic botany, and a lab coordinator for plant biology. Her current research interests focus on algal stress physiology and biochemistry, and the generation of environmentally friendly, alternative fuel sources from green algae. Andi developed two case studies: Evolution of Tungara Frog Mating Calls and Plant Genetic Modification. She also authored learning activities for the Evolution and Phylogenetics, Microbial Diversity, Genetics of Development, Human Evolution, Neurobiology, and Biodiversity units.

Norman A. Johnson, PhD, (see biography under online author) also contributed to the learning activities for the Evolution and Phylogenetics, Microbial Diversity, Genetics of Development, Human Evolution, Neurobiology, and Biodiversity units.

Project Team

Advisors

In addition to determining the content of the units, our advisors and consultants have been actively involved in reviewing the material for all thirteen units throughout its development. Videos, animations, case studies, and text chapters have all been reviewed several times during their production for accuracy and to ensure that these materials are as useful as possible to the intended audience.

Our primary advisors and consultants consisted of a team of eight scientists involved in teaching, curriculum development, and research.

Mark Bloom, PhD, is a science educator at Biological Sciences Curriculum Study (BSCS). He has developed print and Web-based curriculum materials for students in middle school, high school, and college. Previously, he was the assistant director of the Dolan DNA Learning Center, where he ran workshop programs for high school and college teachers. He developed the first educational kits using the polymerase chain reaction and coauthored the college lab manual *Laboratory DNA Science*. Mark was lead advisor for the Genomics, Proteins and Proteomics, Cell Biology and Cancer, and Biology of Sex and Gender units.

Steve Boyarsky is the coordinator of curriculum improvement at Staff Development at Southern Oregon Education Service District. Steve coordinates professional development in a three-county region in southern Oregon. He taught high school biology and human anatomy/physiology for 18 years. Steve has been involved with state and national level biology education through the National Science Teachers Association, a congressional fellowship, grants, and curriculum projects. Steve commented on appropriateness of content, level, and style of all project components.

Alan Dickman, PhD, is the biology curriculum director and an associate professor of biology at the University of Oregon. He has organized summer outreach programs in science for middle school, high school, and community college teachers, and has been involved in nationally funded programs to improve college-level biology education. Alan teaches introductory biology courses and an upper-division forest biology course. As lead scholar, Alan was responsible for final scholarly quality of all content of all project components.

Marion Field Fass, ScD, is an associate professor of biology at Beloit College. She has been involved in curriculum reform efforts in biology through the BioQUEST Curriculum Consortium and the SENCER (Science Education for New Civic Engagements and Responsibilities) project of AAC&U. In 2002 she traveled to Kenya and Tanzania to work with professors who were developing undergraduate courses about the epidemic of HIV/AIDS and about its impact in their communities. Marion was lead advisor for the Microbial Diversity, Emerging Infectious Diseases, HIV and AIDS, and Genetically Modified Organisms units.

Paula Henderson has taught biology at Newark High School in Newark, Delaware since 1980, and received the Outstanding Biology Teacher award for Delaware in 1993. She has taught a course in human heredity and development at the University of Delaware, and is a coauthor of the NIH/BSCS module "The Brain: Understanding Neurobiology Through the Study of Addiction." Paula commented on appropriateness of content, level, and style of all project components.

Patrick Phillips, PhD, is an associate professor of biology and a member of the Center for Ecology and Evolutionary Biology at the University of Oregon. His research focuses on theoretical and empirical studies of evolutionary genetics. He teaches foundations of biology, evolution, population genetics, and experimental design; and is the creator of the evolutionary biology Web site, EvoNet.org. Patrick was lead advisor for the Evolution and Phylogenetics, Genetics of Development, Human Evolution, Neurobiology, and Biodiversity units.

John Postlethwait, PhD, is a professor of biology in the Institute of Neuroscience at the University of Oregon. His research interest is in developmental genetics; he and his group have discovered a genome duplication event that occurred before the vast radiation of teleost fish, which account for half of all species of vertebrates. His lab is currently investigating the genetic mechanisms that may help account for that explosion of biodiversity. The author of two non-majors textbooks for college students, John is committed to undergraduate education and has taught introductory biology to mostly non-biology majors since 1964. John provided critical assistance for the Genetics of Development unit and parts of several other units.

Carol Wheeler is a biology teacher and department chair at Pine Creek High School in Colorado Springs, Colorado. She worked in medical research and was a certified histocompatibility technologist prior to teaching. She received a Christa McAuliffe grant to develop a molecular biology course, and an Intel grant designed to help get students eligible to compete in science fairs at the international level. Carol commented on appropriateness of content, level, and style of all project components.

Evaluation

In addition to the guidance from our team of advisors and consultants, an independent formative evaluation of three of the thirteen units was conducted by **RMC Research Corporation**. RMC Research staff selected ten biology teachers and ten professional development providers, who varied with respect to geographic location, race and ethnicity, and background knowledge in biology. These reviewers provided helpful input on these three units while they were being developed; suggestions made on these units were generalized, where appropriate, to the other ten units.

Funder

Rediscovering Biology is funded by Annenberg/CPB, a partnership between the Annenberg Foundation and the Corporation for Public Broadcasting (CPB), which uses media and telecommunications to advance excellent teaching in American schools. Annenberg/CPB videos help teachers increase their expertise in their fields and improve their teaching methods. For information on obtaining Annenberg/CPB materials, go to www.learner.org or call 1-800-LEARNER.

Producer

Oregon Public Broadcasting (OPB) is a highly experienced producer of educational content with expertise in both traditional and new media approaches to formal education, community outreach, and television production.

OPB has produced many series for Annenberg/CPB, including *THE UNSEEN UNIVERSE: An Introduction to Microbiology; A WORLD OF ART: Works in Progress,* a series on contemporary artists; *AMERICAN PASSAGES: A Literary Survey*, a multimedia series for college students; and *ARTIFACTS & FICTION*, a professional development workshop series for teachers on interdisciplinary approaches to American literature. OPB has also been the co-producer for video series and digital materials to accompany several McGraw-Hill textbook publications.

OPB has a long history of producing Web sites, teachers' guides, and other curriculum materials to accompany educational and PBS broadcast series. Working in close concert with national advisory boards, OPB's staff has produced curriculum materials in the humanities and sciences for a variety of grade levels and teacher professional development.

OPB is also a major producer of PBS Primetime documentary series, and has created programming for NOVA, FRONTLINE, and other programs as well as numerous specials and limited series.

Research Staff

Rediscovering Biology would not be possible without the hard work of the research and production staff at Oregon Public Broadcasting. The research staff provided critical support for video producers, authors, and activity developers.

Cindy Lefton has a bachelor's degree in zoology and a master's degree in mass communication with an emphasis on science writing and editing. She has served as the editor of a medical news magazine, and has edited several medical textbooks and journal articles. Her interests in science and nature have lead to volunteer service as an education coordinator for a wildlife rehabilitation facility, a zoo guide, and a science fair coordinator.

Liza Nicoll earned a bachelor's degree in biology and a bachelor's degree in health science at the University of Massachusetts at Amherst in the spring of 2001. Since completing work on *Rediscovering Biology* she has continued to work in television production, researching for a world history documentary series.

Stephani Sutherland earned a doctorate in neuroscience from the Vollum Institute at Oregon Health & Science University, where she coordinated an outreach program in public junior high and high schools called Kids Interested in Discovering Science (KIDS). Since leaving the research laboratory in 2001, she has worked as a science news reporter for the Los Angeles Times and traveled around the world. She now works for the Journal of Neuroscience and writes freelance science news for various journals. Stehpani was also a coauthor for the Neurobiology chapter of the online text.

Interviewees

We are grateful to so many of these people who were willing to find time for this project. The following people provided valuable information to the project through interviews.

Genetically Modified Organisms

Leon Corzine; David L. Dornbos, Jr., PhD; Rebecca J. Goldburg, PhD; Marion Nestle, PhD, MPH; Thomas E. Newberry; and Gary H. Toenniessen, PhD.

Emerging Infectious Diseases

Capt. Daniel Carucci, MD, PhD; Rita Colwell, PhD; Laurie Garrett; Stuart B. Levy, MD; Judith M. Martin, MD; and Lukas K. Tamm, PhD.

Cell Biology Cancer

Elizabeth Blackburn, PhD; Brian Druker, MD; Leland Hartwell, PhD; Mary-Claire King, PhD; and Robert Weinberg, PhD.

Biology of Sex and Gender

Holly Ingraham, PhD; David Page, MD; and Eric Vilain, MD, PhD.

Genomics

David Altshuler, MD, PhD; James Carrington, PhD; Jonathan Eisen, PhD; and Eric Lander, PhD.

Proteins and Proteomics

Hamid Bolouri, PhD; Ned David, PhD; Stanley Fields, PhD; Hunter Fraser; Aaron Hirsh; and Leroy Hood, PhD.

Microbial Diversity

Anne Camper, PhD; Bill Costerton, PhD; Dan Kotansky, PhD; Anna-Louise Reysenbach, PhD; Frank F. Roberto, PhD; Phil Stewart, PhD; and Paul Sturman.

HIV and AIDS

Edward Berger, PhD; Laurie Garrett; Jay Levy, MD; Rob Roy MacGregor, MD; Erik Vonmuller; and David Weiner, PhD.

Evolution and Phylogenetics

Phillip Gingerich, PhD; Timothy Read, PhD; and Carl Woese, PhD.

Human Evolution

Kari Stefansson, MD; Ian Tattersall, PhD; Ajit Varki, MD; and Christopher Wills, PhD.

Neurobiology

Wolfhard Almers, PhD; Fred Gage, PhD; Richard Huganir, PhD; and John Williams, PhD.

Biodiversity

James Miller, PhD; Richard Ostfeld, PhD; Peter H. Raven, PhD; Eleanor Sterling, PhD; and G. David Tilman, PhD.

Genetics of Development

Judith Eisen, PhD; Markus Grompe, MD; John Incardona, PhD; Nipam Patel, PhD; and John Postlethwait, PhD.

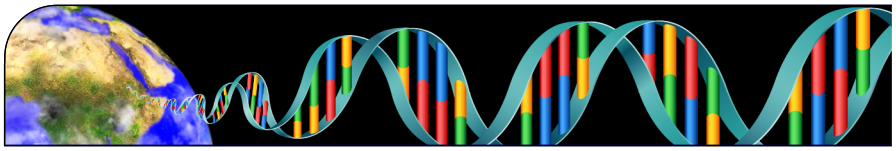
Additional Acknowledgements

We would like to thank the following people at Oregon Public Broadcasting in addition to the researchers who made this project possible.

Executive Producer Meighan Maloney; Production Manager Doug Brazil; Production Media Manager Catherine Stimac; Production Assignment Manager Joshua Wolfe; Web Developer John Kin; Web Assistant Ryan Servatius; Assistant Production Manager Mary Hager; Database Administrator Heather Chambers; and Copyeditor Jennifer Ingraham.

The *Rediscovering Biology* video series was produced by Oregon Public Broadcasting's Educational Media Production Department. The creative team consisted of the following: Executives in Charge of Production David Davis and Jack Galmiche; Executive Producer Meighan Maloney; Producer/Writers Melissa Gerr, Nadine Jesling, Amanda Lowthian, and Eric Slade; Writer Andrew Holtz; Series Host Lew Frederick; Academic Director Alan Dickman; Production Assignment Manager Joshua Wolfe; Production Manager Doug Brazil; Production Media Manager Catherine Stimac; Researchers Cindy Lefton, Liza Nicoll, and Stephani Sutherland; Director of Production Services Milt Ritter; Manager of Production Scheduling Bill Dubey; Director of Engineering Information Dave Fulton; Assistant Director Sean Hutchinson; Assistant Production Manager Mary Hager; Pre-Production Coordinator Thea Bergeron; Videographers Art Adams, Karel Bauer, S.O.C., David Dennison, Paul Jacobson, Lisa Suinn Kallem, Jim Langley, Michael McNamara, Corky Miller, Ben Nieves, John Patzer, Todd Sonflieth, Dave Spangler, and Wally Szczubialk; Editors Tom Babich, Bruce Barrow, Sarah Marcus, Chris Nolan, John Patzer, and Kate Schoninger; Field Audio Michael A. Bidese, Chad Birmingham, Darren Brower, Kevin Brown, Chris Callus, Francis X. Coakley, Tony D'Annunzio, Thom Dentler, Jay Farrington, Dave Foreman, Thomas Forliti, Gerry Formicola, G. John Garrett, Joel Groeblinghoff, Cindy Hogan, Chip Lake, Randy Layton, Gordon Masters, Casey Quinlan, C.A.S, Todd Schmidt, Brandt Sennhenn, Mike Tyrey, Ted Ver Valen, Bill Ward, and Matt Yeasley; Creative Director Tim Bergmann; Production Artists Dora Papay, Corrina Reff, and Jefferson P. Vowell; 3-D Animations Hot Pepper Studios, Animation Dynamics, Inc., and Kevin Washington; Rights Assistant Morgan Currie; Theme Music Cal Scott; Production Intern Larry Johnson; Production Art Interns Soumalay Douangmala, Kim Harshberger, and Kevin Jaquette; Production Assistants Michael Aaris, David Banyan, Emily Chapman, Mike Forest, Kenyatta Gomez, Madeleine Pappas, Michelle Pridemore, Anastasia Savko, Alex Selkowitz, and Jonathan Zintel.

The *Rediscovering Biology* Web Site was produced by the following creative team: Oregon Public Broadcasting Web Developer/Producer John Kin; Web Assistant Ryan Servatius; Database Administrator Heather Chambers; Project Coordination, Flash Interactive Development and Project Design AMAZING! Online Marketing, LLC in association with Moshofsky/Plant Creative Services and Bergmann Graphics.



REDISCOVERING BIOLOGY

Molecular to Global Perspectives

Genomics

"…the acquisition of the sequence is only the beginning. The sequence information provides a starting point from which the real research into the thousands of diseases that have a genetic basis can begin." J. Craig Venter[1]

The Human Genome Project

In 1986 Nobel laureate Renato Dulbecco laid down the gauntlet to the scientific community to sequence the complete human genome. "Its significance," he said, "would be comparable to that of the effort that led to the conquest of space, and it should be carried out with the same spirit."[2] Dulbecco also argued that such a project should be "an international undertaking, because the sequence of the human DNA is the reality of the species, and everything that happens in the world depends upon those sequences."

Like the conquest of space, sequencing the human genome required the development of wholly new technologies. The human genome, containing more than three billion nucleotides, is vast. In 1986 DNA sequencing had yet to be automated and, consequently, was slow and tedious. Moreover, computer software for sequence analysis was just being developed. Similar to the Apollo project that met President Kennedy's goal of a manned lunar landing by 1970, the genome project also succeeded — beyond the dreams of the scientists who proposed it.

During the 1990s rapid progress was made in developing automated sequencing methods and improving computer hardware and software. By 2003 biologists had sequenced genomes from about one hundred different species. These species included dozens of bacteria and other microbes, as well as the model systems: yeast, fruit fly, nematode, and mouse. The capstone, of course, was the completion of the human genome sequence. In 2001 two rival teams jointly announced the completion of a draft sequence of the entire human genome, consisting of more than three billion nucleotides.

Is human DNA "the reality of the species"? Do we now have all the information we need to define human life? Perhaps surprisingly, the answers are no. Genetics is more than just DNA. While DNA is the blueprint for life, proteins carry out most cellular functions; DNA just codes for RNA, which codes for protein.

One major surprise emerged from the sequencing of the human genome. Although some scientists expected to find at least 100,000 genes coding for proteins, only about 30,000–35,000 of such genes

appear to be in the human genome. These genes comprise only about two percent of the entire DNA. What is the rest of the DNA doing? Biologists once thought that this noncoding DNA was just junk, and hence called it "junk DNA." As we will see below, evidence now suggests that some junk DNA may have functions.

The quest to understand the workings of human cells will not be over until we understand how this genetic blueprint is used to produce a particular set of proteins — the proteome — for each type of cell and how these proteins control the physiology of the cell. (See the *Proteins and Proteomics* unit.) We should think of the human genome as a database of critical information that serves as a tool for exploring the workings of the cell and, ultimately, understanding how a complex living organism functions.

Sequencing a Genome

Sequencing a genome is an enormous task. It requires not only finding the nucleotide sequence of small pieces of the genome, but also ordering those small pieces together into the whole genome. A useful analogy is a puzzle, where you must first put together the pieces of a smaller puzzle and then assemble those pieces into a much larger picture. Two general strategies have been used in the sequencing of large genomes: clone-based sequencing and whole genome sequencing (**Fig. 1**).

In **clone-based sequencing** (also known as *hierarchical shotgun sequencing*) the first step is mapping. One first constructs a map of the chromosomes, marking them at regular intervals of about 100 kilobases (kb). Then, known segments of the marked chromosomes (which can contain very small fragments of DNA) are cloned in **plasmids**. One special type of plasmid used for genome sequencing is a **BAC** (bacterial artificial chromosome), which can contain DNA fragments of about 150 kb. The plasmid's fragments are then further broken into small, random, overlapping fragments of about 0.5 to 1.0 kb. Finally, automated sequencing machines determine the order of each nucleotide of the many small fragments.

Data management and analysis are critical parts of the process, as these sequencing machines generate vast amounts of data. As the data are generated, computer programs align and join the sequences of thousands of small fragments. By repeating this process with the thousands of clones that span each chromosome, researchers can determine the sequences of all the larger clones. Once they know the order of all the larger clones, the researchers can join the clones and determine the sequence of each chromosome.

Finding the sequence of the smaller clone fragments is relatively easy. The challenge is assembling all the pieces. The National Human Genome Research Institute (the public consortium headed by Francis Collins) used clone-based sequencing for the human genome. In doing so, they relied heavily on the work of computer scientists to assemble the final sequence.

Whole genome shotgun sequencing skips the mapping step of clone-based sequencing. Instead, it (1) clones millions of the genome's small fragments in plasmids, (2) sequences all of these small overlapping fragments, and then (3) uses computers to find matches and join them together.

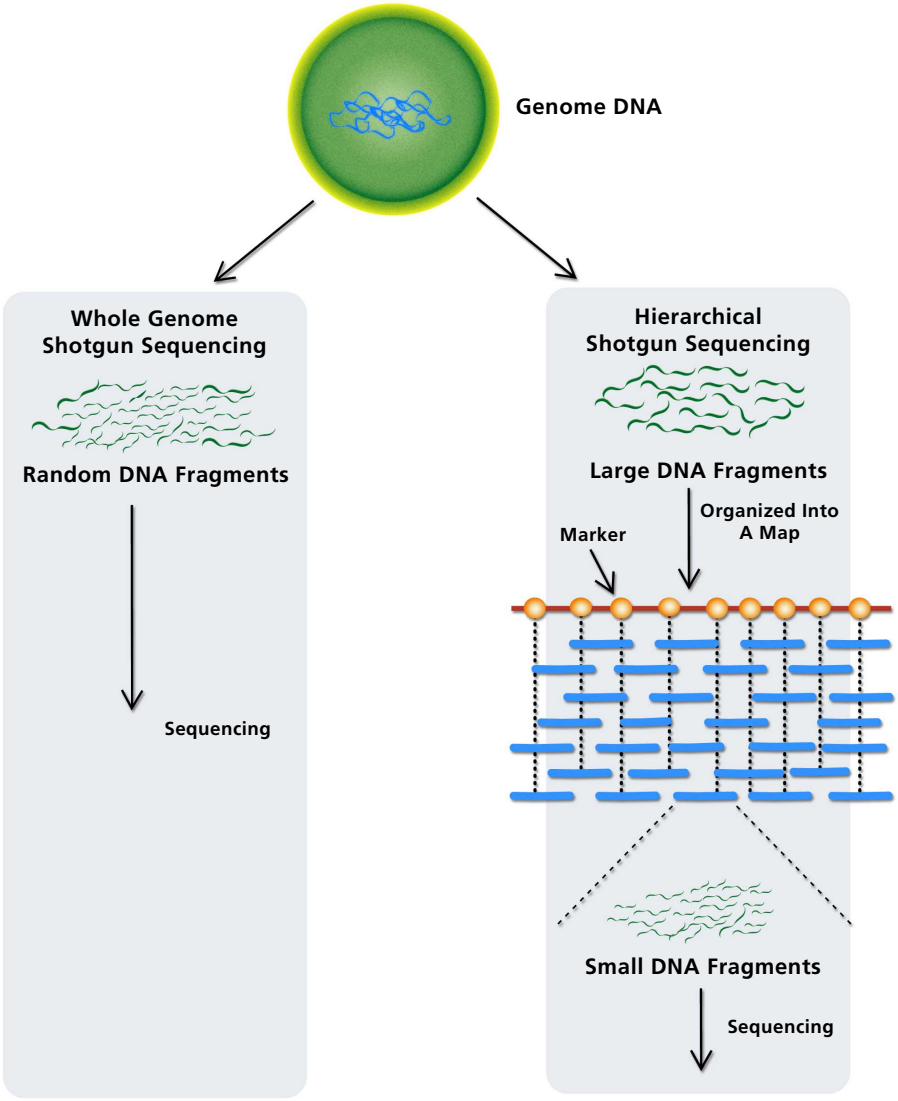


Illustration adaptation — Bergmann Graphics

Figure 1.
Strategies for cloning whole genomes.

. . . . . AGTCTAGCCGTAGCTATTATGCAG
TATTATGCAGATGGCACCTAGGA . . . . . .

Assembly

. . . . . AGTCTAGCCGTAGCTATTATGCAGATGGCACCTAGGA . . . . . .

Celera Genomics, a private company headed by J. Craig Venter, used this approach to clone the human genome. Although they started much later than the public consortium, Celera completed its draft sequence at about the same time as the consortium; however, it had the advantage of having access to all the consortium's maps.

Genome sequencing projects now generally use some combination of chromosome mapping, and clone-based and whole genome shotgun sequencing of smaller fragments. The technology developed for sequencing the human genome — both in terms of sequencing DNA and in the software and hardware used to assemble the sequences into a genome — has resulted in the rapid sequencing of many other genomes.

Finding Genes

Imagine the genome as an encyclopedia with a volume for each chromosome. If you were to open a volume, you would find page after page containing only four letters — A, T, G, and C — without spaces or punctuation. How could you read such a book, or even identify possible words and sentences? The genome sequence itself does not provide direct information on the location of a gene, but there are clues embedded in the sequence that computer programs can find.

Most simple gene prediction programs use several pieces of sequence information to identify a potential gene in a DNA sequence. The programs look for sequences in the DNA that have the potential to encode a protein. These sequences are called **open reading frames (ORFs)**. An ORF usually begins with a codon of UAG (**Fig. 2**), and then contains a long sequence of codons that specify the protein's amino acids. The ORF then ends with a stop codon of UAA, UAG, or UGA. Using overlapping frames of three nucleotides each, the computer program searches the database until it identifies an ORF region. For example, the sequence "abcdefghijk" could be read in three-letter "words" of "abc-def-ghi," "bcd-efg-hij," or "cde-fgh-ijk." Computer programs can scan DNA sequences quickly, using these overlapping reading frames on both the original strand and on the complementary strand, producing a total of six different reading frames for any sequence.

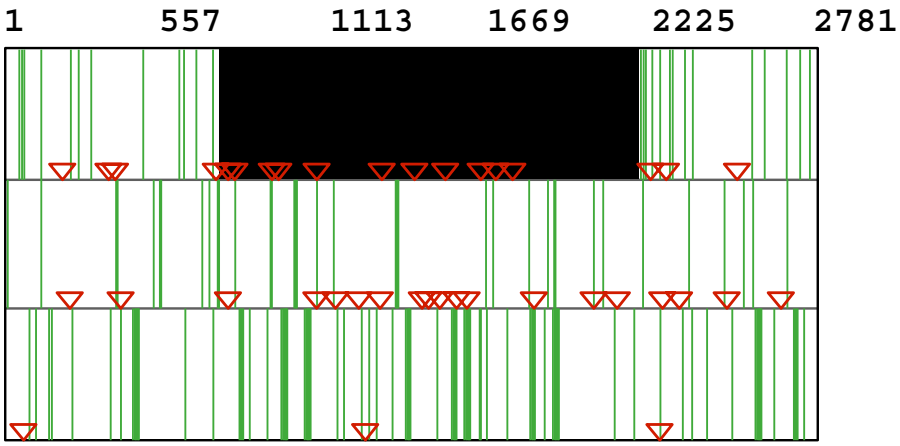


Figure 2. To find an open reading frame (ORF), a computer program identifies start codons (red arrows) and stop codons (green lines) in all three reading frames (represented by the three stacked rows). The black box is the largest ORF found in this sequence.

Using these programs to find ORFs in bacterial genomes is relatively easy. Here, the DNA sequence matches the mRNA. The situation is more complicated for eukaryotic genes, which often contain one or more noncoding regions (**introns**). To find ORFs in these genes, the introns are removed in a process called *splicing* (**Fig. 3**). The final spliced mRNA, which encodes the protein product of the gene, is smaller than the original RNA transcript that matches the genome. The introns are removed, leading to the splicing of the coding regions of a gene (**exons**) together into the final mRNA. The problem is that a simple ORF-finding program cannot be used with genomic DNA that has introns because those genes do not match the mRNA. While computer programs can identify eukaryotic genes with introns, they are not always accurate.

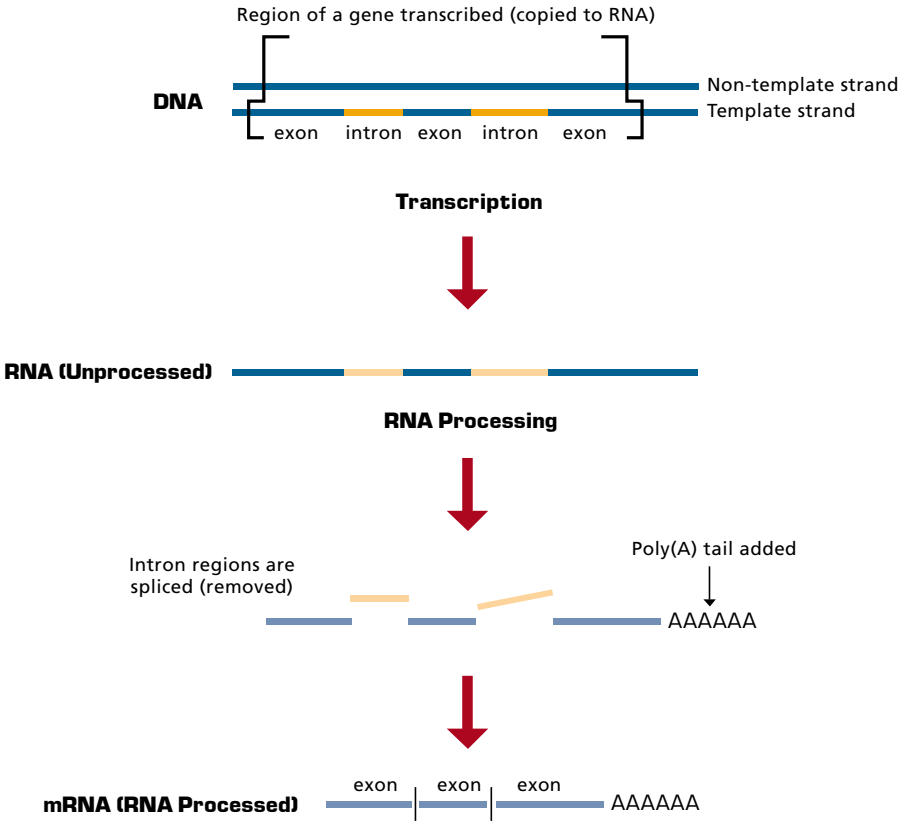


Figure 3. A gene consists of coding regions, called exons, that are interrupted with intervening noncoding regions, called introns. During transcription, the whole segment of DNA that corresponds to a gene is copied to make RNA. During RNA processing, the introns are removed and the exons are joined. A poly(A) tail is added to the mRNA.

An alternate approach to characterize genes in eukaryotes is to first make a DNA copy of the mRNA encoded by the gene. To do this, one uses an enzyme called *reverse transcriptase*. The copy, called **cDNA** or *complementary DNA*, has the same sequence as the mRNA, except that the U is replaced by a T. Because the cDNA lacks introns, the sequence of the cloned cDNA can be used to find an ORF. In addition to simply identifying ORFs, many advanced sequence analysis programs use other information to help identify eukaryotic genes in the chromosome. (See the BLAST section below.)

Is the Eukaryotic Genome a Vast Junkyard?

Bacteria have small, compact genomes, rich in genes. These genes have fewer noncoding regions and no introns. Eukaryotic genomes, however, often have much more DNA content than prokaryotic genomes. While eukaryotes generally have more genes than bacteria, the difference in gene content is not as great as the difference in DNA content: there is much more noncoding DNA in eukaryotes. In fact, gene-coding regions comprise only about two percent of the human genome.

Most eukaryotic genes are interrupted by large introns. Even with these introns included, however, genes comprise only about twenty-five percent of the human genome. In eukaryotes, repeated sequences characterize great amounts of noncoding DNA. Some of this repetitive DNA is dispersed more or less randomly throughout the genome. There are also millions of copies of other, shorter repeats, but they are typically found in larger blocks. Some trinucleotide (3 bp) repeats are associated with diseases such as fragile X and Huntington's disease, which result from extra copies of the repeat sequence.

Most of these repeat sequences are **transposable elements**, that can replicate and insert a copy in a new location in the genome. The result is the amplification of these repetitive elements over time. Transposable elements can be harmful because they can cause mutation when they move into a gene. They also use cellular resources for replication and expression. Are these elements unwelcome guests gone wild or may they actually be useful components of the genome? We don't really know, but there are some tantalizing suggestions of functions for some of these elements. About one million copies of the repetitive DNA element called *Alu repeats* lurk in the genomes of each one of us. What are they doing? One study found that these bind to proteins used to reshape chromatin during cell division. Perhaps this apparent junk DNA is actually helping provide structure to the chromosome and regulate the production of proteins in different cell types.

Genomes differ in size, in part because they have different proportions of repetitive DNA. For example, the total genome size of the puffer fish is about one-tenth the size of the human genome. However, the puffer fish genome has about the same number of genes as the human genome, and the genes appear to have the same functions. The puffer fish genome is also smaller than the human genome. This is partially because it contains only about fifteen percent repetitive DNA, while more than half the human genome is repetitive DNA. Because most human genes are present in the puffer fish and the puffer fish genome is less cluttered by repetitive DNA, this model organism may help scientists identify the genes responsible for human diseases.

The Difference May Lie Not in the Sequence but in the Expression

Most genes are shared across all animals. More than ninety-nine percent of human genes have a related copy in the mouse. As one examines animals that are more distantly related, the proportion of the genes they share decreases; however, despite about 500 million years of evolutionary separation, half the genes in the lowly sea squirt

correspond to those found in humans. This remarkable conservation of gene structure is striking considering how much these animals differ in morphology, physiology, and behavior.

If they share so many of the same genes, why are different animals so different? Differences among species result largely from differences in the time and location of the genes' expression. Let us consider our closest relative, the chimpanzee. Not only do chimpanzees and humans share nearly all of the same genes, but the DNA sequences of those genes also are very similar between the two species. Svante Pääbo sequenced three million bases of the chimp genome and found that chimps and humans differ overall by less than two percent at the sequence level. (See the *Human Evolution* unit.) Based on the low sequence divergence, Pääbo hypothesized that the difference between humans and chimpanzees was due mainly to how the genes were expressed in the different species.

To test this hypothesis, Pääbo compared the expression pattern of 20,000 human genes in humans and chimps. He found that while expression levels were similar in liver cells and blood, there were larger differences in brain cells. This suggests that the human brain has increased the use of certain genes compared to those same genes in a chimp. So, it not so much the sequence of the genes that is important, but how they are expressed to make the cell's proteins that determines the unique characteristics of each organism.

Determining Gene Function from Sequence Information

Researchers have produced an enormous number of genome sequences from a variety of organisms. Publicly available databases, such as GenBank at the NCBI (National Center for Biotechnology Information), store many of these sequences. The databases have been a tremendous boon for comparative biology. The NCBI database stores not only the genome sequences, but also information about the function (if it is known) of the genes.

The NCBI can also identify unknown genes by comparing them with known genes in the database. One program commonly used for this purpose is **BLAST** (Basic Local Alignment Search Tool). Sequence similarity searching algorithms like BLAST are based on the premise that if two sequences are similar then they are likely to be **homologous** (that is, they share a common evolutionary ancestor). (See the *Evolution and Phylogenetics* unit.) Using this database, one can infer the function of an unknown gene by finding similar sequences of known genes and proteins. For example, suppose you were to use BLAST to search for sequences similar to a new gene. Upon viewing your results, you noticed that all the sequences with a high degree of similarity to the new gene belonged to a family of genes known to break down hydrogen peroxide. You could logically conclude, then, that this new gene encoded a protein with a similar function.

BLAST searches can be done at the nucleotide level; however, comparisons at the amino acid level provide much greater sensitivity. Therefore, unless one is particularly interested in the DNA sequence itself, it is better to search for genes using protein. If you have only raw nucleotide sequence data, computer programs can automatically

translate the DNA into amino acids using all six reading frames (three frames from one strand and three frames from the complementary strand) before searching the protein database.

In addition to whole proteins, similarity searches can identify **protein motifs**. A motif is a distinctive pattern of amino acids, conserved across many proteins, which gives a particular function to the protein. For example, the presence of one particular motif in a protein indicates that this protein probably binds ATP and may therefore require ATP for its action.

The result of a database search is a list of matches, ranked from highest to lowest, based on the probability of a significant match (**Fig. 4**). The reported alignment scores are given "expectation values" (E), which represent the probability that a match with the reported score would be expected to occur by random chance. The smaller the E-value, the higher the assigned score and the less likely that the match was coincidence. Some of the easiest results to interpret are very high scores (small E-values, low-probability), which usually result from two very similar proteins. Other easily identifiable results are very low scores, which indicate that the outcome is probably the result of chance similarity.

```
                                                                  Score    E
Sequences producing significant alignments:                       (bits) Value

gi|4504351|ref|NP_000510.1|  delta globin [Homo sapiens] >gi...   128   2e-29
gi|70353|pir||HDHU  hemoglobin delta chain - human                128   2e-29
gi|122714|sp|P02043|HBD_PANTR  Hemoglobin delta chain >gi|34...   128   2e-29
gi|70354|pir||HDCZ  hemoglobin delta chain - chimpanzee (ten...   127   2e-29
gi|18462105|gb|AAL72117.1|  delta-globin [Homo sapiens]           127   5e-29
gi|229172|prf||640488A  hemoglobin delta                          125   2e-28
gi|122584|sp|P02028|HBB_CERAE  Hemoglobin beta chain >gi|703...   122   1e-27
gi|183855|gb|AAA52635.1|  hbbm fused globin protein ( beta c...   122   1e-27
gi|122668|sp|P02032|HBB_PREEN  Hemoglobin beta chain >gi|703...   122   2e-27
gi|122616|sp|P02025|HBB_HYLLA  Hemoglobin beta chain >gi|703...   121   2e-27
gi|223012|prf||0404170B  hemoglobin beta                          121   2e-27
gi|122712|sp|P19886|HBD_COLPO  Hemoglobin delta chain >gi|86...   121   3e-27
gi|4929993|pdb|1CH4|A  Chain A, Module-Substituted Chimera H...   121   3e-27
gi|122636|sp|P08259|HBB_MANSP  Hemoglobin beta chain >gi|703...   120   3e-27
gi|13549112|gb|AAK29639.1|AF349114_1  beta globin chain vari...   120   3e-27

 >gi|122668|sp|P02032|HBB_PREEN  Hemoglobin beta chain
  gi|70332|pir||HBMQP  hemoglobin beta chain - hanuman langur
          Length = 146

 Score =  122 bits (305), Expect = 2e-27
 Identities = 57/60 (95%), Positives = 57/60 (95%)

Query: 1  VHLTPEEKTAVNALWGKVNVDAVGGEALGRLLVVYPWTQRFFESFGDLSSPDAVMGNPKV 60
          VHLTPEEK AV ALWGKVNVD VGGEALGRLLVVYPWTQRFFESFGDLSSPDAVMGNPKV
Sbjct: 1  VHLTPEEKAAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSSPDAVMGNPKV 60
```

Figure 4. The results of a BLAST search using the delta chain of hemoglobin as the query.

Search results also provide links (in blue) to a database page with information on each sequence similar to the query sequence. This page gives extensive information on the match sequence, including the organism it came from, the function of the gene product (if it is known), and references to journal articles concerning the sequence. BLAST results also provide the actual alignment results for nucleotides or amino acids between the query sequence and the match sequences.

The Virtues of Knockouts

Gene prediction programs have been valuable in the preliminary identification of genes; however, they have limitations. Unless the gene of interest is homologous to a gene of known function, the function is generally still not known. A biological approach to determining the function of a gene is to create a mutation and then observe the effect of the mutation on the organism. This is called a **knockout study**. While it is not ethical to create knockout mutants in humans, many such mutants are already known, especially those that cause disease. One advantage of having a genome sequence is that it greatly facilitates the identification of genes in which mutations lead to a particular disease.

The mouse, where one can make and characterize knockout mutants, is an excellent model system for studying genetic diseases of humans; its genome is remarkably similar to a human's. Nearly all human genes have homologs in mice, and large regions of the chromosomes are very well conserved between the two species. In fact, human chromosomes can be (figuratively) cut into about 150 pieces, mixed and matched, and then reassembled into the 21 chromosomes of a mouse. Thus, it is possible to create mutants in mice to determine the probable function of the same genes in humans. Genetic stocks of mutant mice have been developed and maintained since the 1940s.

One goal of the mouse genome project is to make and characterize mutations in order to determine the function of every mouse gene. After a particular gene mutation has been linked to a particular disorder, the normal function of the gene may be determined. An example of this approach is the mutated gene that resulted in cleft palates in mice. The researchers found that the gene's normal function is to close the embryo's palate. An understanding of the genetics behind cleft palate in mice may one day be used to help prevent this common birth defect in humans.

Genetic Variation Within Species and SNPs

A **polymorphism**, the existence of two or more forms of sequence between different individuals of the same species, can arise from a change in a single nucleotide. These **single nucleotide polymorphisms** (**SNPs**) account for ninety percent of all polymorphisms in humans. The number of SNPs between two genomes provides a measure of sequence variation; however, the variation is not uniform over the genome. About two-thirds of SNPs are in noncoding DNA and tend to be concentrated in certain locations in the chromosome. In addition, sex chromsomes have a lower concentration of SNPs than autosomes.

There are about three million SNPs in the human genome, or about 1 per 1000 nucleotides. SNPs are ideal genetic markers for many applications because they are stable, widespread, and can often be linked to particular characteristics (phenotypes) of interest. They are proving to be among the most useful human markers for studies of evolutionary genetics and medicine.

Not all SNPs, even when they are present in coding genes, lead to visible or phenotypic differences among individuals. Changes in the DNA sequence don't always change the amino acid sequence of the protein. For example, a change from GGG to GGC results in no change in the protein because both codons result in a glycine in the protein. This is called a **synonymous mutation** or *silent mutation*; non-synonymous substitutions do cause a change in the amino acid. About half of all SNPs in genes are non-synonymous and therefore can account for diversity between individuals or populations. Depending on the particular change in an amino acid caused by a nonsynonymous mutation, the resulting protein may be an active, inactive, or partially active. It may also be active in a different way.

One well-characterized SNP exists in a gene in chromosome 6. Individuals with cysteine at amino acid position 282 are healthy; however, about 1 in 200–400 Caucasians of Northern European descent possess two copies of that gene where the amino acid is tyrosine instead of cysteine. Due to this one change, these individuals have a disease called *hereditary hemochromatosis*. People afflicted with this disease accumulate high levels of iron, which causes permanent damage to the organs, especially the liver. About ten percent of these individuals carry only one copy of this mutation; they are heterozygous and are carriers of the disease. A genetic test for hereditary hemochromatosis is available, which can detect the SNP. If the disease is found, medical professionals can then determine whether the person is homozygous or heterozygous for this allele. Another example of a single SNP that has a dramatic effect is the one that leads to sickle cell anemia. (See the *Human Evolution* unit.)

Identifying and Using SNPs

In order to identify SNPs, nucleotide sequences of two or more genomic regions must be aligned so that the polymorphisms are apparent. Sequence alignments are easy when the sequences are similar, but can be very difficult when there are many polymorphisms. The alignment of two sequences is determined by a program that compares the two sequences, nucleotide by nucleotide. For multiple sequences, the program continues the same type of pairwise alignment for all possible pairs. The result is a pairwise distance matrix based on all possible alignments of any two sequences. This matrix is then used to construct a phylogenetic tree that predicts how closely related two sequences are, based on their similarity. The program then uses this information to align the sequences, again in order of their relatedness. This is the method used in a program called **CLUSTAL**. A typical output from CLUSTAL is shown in **Figure 5.**

Figure 5. A CLUSTAL alignment of a segment of a gene from four species. The red letters show the amino acid sequence (R=arginine, P=proline, G=glycine, etc.). The nucleotides that are conserved in all four species are shown in the columns with an asterisk at the bottom.

```
          R  P  P  G  K  S  G  K  Y  Y  Y  Q  L  N  S  K  K  H  H    159
Human     CGGCCGCCGGGCAAGAGCGGCAAGTACTACTACCAGCTCAACAGCAAGAAGCACCAC  642
Mouse     CCCCCGCCAGG-AAGAGCGGCAAGTATTATTATCAGCTAAATAGCAAAAAGCACCAC  614
Chicken   CAGTCCCACAGCAAG---GGCAAGTACTACTACCAGCTCAACAGCAAGAAGCACCAC  583
Frog      CATTCCAGTAACAAG---AAAAAATACTATTATCAGCTCAATAGCAAAAAACATCAT  500
          *    *        ***       ** ** ** ** ***** ** ***** ** ** **
```

Most SNPs have no effect on an individual, so what use are having maps of them? SNPs appear to cluster in blocks called **haplotypes**. Grouping individuals that share a particular haplotype is called *haplotyping*. Because these particular sequences of SNPs on a chromosome are inherited together as blocks, they can be used to distinguish individuals and populations. What good is haplotyping? One can determine what specific diseases or other traits are associated with different haplotypes. In most cases, there are much fewer haplotypes than SNPs. Although it is the SNPs that actually cause disease, looking for changes in one SNP out of millions in the genome is not practical; looking for a particular haplotype is much easier.

An example of the value of haplotype comes from research on Crohn's disease. Crohn's disease is a chronic inflammatory disease of the digestive tract that tends to cluster in families. Researchers identified a haplotype on chromosome 5 that correlates with the disease. This region of the chromosome contains genes involved in immunity; these genes then may be important in other inflammatory diseases, such as lupus or asthma.

Practical Applications of Genomics

Genome sequence data now provide tools for the development of practical uses for genetic information. DNA is an invaluable tool in forensics because — aside from identical twins — every individual has a uniquely different DNA sequence. Repeated DNA sequences in the human genome are sufficiently variable among individuals that they can be used in human identity testing. The FBI uses a set of thirteen **short tandem repeat** (**STR**) DNA sequences for the Combined DNA Index System (CODIS) database, which contains the **DNA fingerprint** or profile of convicted criminals. Investigators of a crime scene can use this information in an attempt to match the DNA profile of an unknown sample to a convicted crimina. DNA fingerprinting can also identify victims of crime or catastrophes, as well as many family relationships, such as paternity. While we think of forensics in terms of identifying people, it can also be used to match donors and recipients for organ transplants, identify species, establish pedigree, or even detect organisms in water or food. (See the *Evolution and Phylogenetics* unit.)

An unusual application of DNA fingerprinting technology is a project of Mary-Claire King's at the University of Washington. (See the *Cell Biology and Cancer* unit.) Although her research is primarily concerned with the identification of genetic markers for breast cancer, she also has a project to help the "Abuelas," or grandmothers, in Argentina. In Buenos Aires in the 1970s and 1980s, children of activists "disappeared" during the military dictatorship. The children were placed in orphanages or illegally adopted when their parents were killed. Now King is using mitochondrial DNA, which is inherited only maternally, to reunite the children with their grandmothers.

The basis of many diseases is the alteration of one or more genes. Testing for such diseases requires the examination of DNA from an individual for some change that is known to be associated with the disease. Sometimes the change is easy to detect, such as a large addition or deletion of DNA, or even a whole chromosome. Many changes are very small, such as those caused by SNPs. Other changes can

affect the regulation of a gene and result in too much or too little of the gene product. In most cases if a person inherits only one mutant copy of a gene from a parent, then the normal copy is dominant and the person does not have the disease; however, that person is a carrier and can pass the disease on to offspring. If two carriers produce a child and each passes the mutant allele to the child (a one-in-four probability), that individual will have the disease.

Several different mutations in a gene often lead to a particular disease. Many diseases result from complex interactions of multiple gene mutations, with the added effect of environmental factors. Heart disease, type-2 diabetes and asthma are examples of such diseases. (See the *Human Evolution* unit.) Many diseases do not show simple patterns of inheritance. For example, the BRCA1 mutation is a dominant mutant allele that leads to an increased risk for breast and ovarian cancer. (See the *Cell Biology and Cancer* unit.) Although not everyone with the mutation develops the disease, the risk is much higher than for individuals without the mutation.

Newborns commonly receive genetic testing. The tests detect genetic defects that can be treated to prevent death or disease in the future. Apparently normal adults may also be tested to determine whether they are carriers of alleles for cystic fibrosis, Tay-Sachs disease (a fatal disease resulting from the improper metabolism of fat), or sickle cell anemia. This can help them determine their risk of transmitting the disease to children. These tests as well as others (such as for Down's syndrome) are also available for prenatal diagnosis of diseases. As new genes are discovered that are associated with disease, they can be used for the early detection or diagnosis of diseases such as familial adenomatous polyposis (associated with colon cancer) or p53 tumor-suppressor gene (associated with aggressive cancers). The ultimate value of gene testing will come with the ability to predict more diseases, especially if such knowledge can lead to the disease's prevention.

Gene therapy is a more ambitious endeavor: its goal is to treat or cure a disease by providing a normal copy of the individual's mutated gene. (See the *Genetically Modified Organisms* unit.) The first step in gene therapy is the introduction of the new gene into the cells of the individual. This must be done using a vector (a gene carrier molecule), which can be engineered in a test tube to contain the gene of interest. Viruses are the most common vectors because they are naturally able to invade the human host cells. These viral vectors are modified so that they can no longer cause a viral disease.

Gene therapy using viral vectors does have a few drawbacks. Patients often experience negative side effects, and expression of the desired gene introduced by viral vectors is not always sufficiently effective. To counter these limitations, researchers are developing new methods for the introduction of genes. One novel idea is the development of a new artificial human chromosome that could carry large amounts of new genetic information. This artificial chromosome would eliminate the need for recombination of the introduced genes into an existing chromosome. Gene therapy is the long-term goal for the treatment of genetic diseases for which there is currently no treatment or cure.

Examining Gene Expression

Understanding the functions of genes depends on knowing when and in what cells they are each expressed. How can one measure the amount of mRNA transcribed from a gene in a particular cell type? The standard method uses a probe — a DNA sequence unique for that gene — which binds to the mRNA that has the complementary sequence. The more mRNA particular cell produces, the more mRNA that is bound to the probe, giving the probe an increased signal. Because cDNA is complementary in sequence to mRNA, it can also be used to measure the expression of a particular gene.

Organisms have so many genes in their genomes that studying the expression of all of these genes had been exceedingly difficult. Going from studying gene expression one gene at a time to examining expression patterns of a multitude of genes required new technology.

In the late 1990s the development of **microarray chips** allowed researchers to examine the expression of thousands of genes simultaneously. This allowed for a much broader perspective of gene expression than was possible when genes were analyzed singularly. Microarray chips are glass slides spotted with many rows containing tiny amounts of probe DNA, one for each of thousands of genes (**Fig. 6**). The target sample of interest, usually made from mRNA of a specific type of cell, is labeled with a fluorescent dye and added to the chip. If there is a match between the sample of interest and the DNA probe on the chip, the two molecules will bind to each other. Then, when exposed to a laser, the spot will produce a signal that will fluoresce. (Figure 6 describes this process in more detail.)

Scientists can use microarrays, a rapid and sensitive test, in a variety of experimental studies. Using microarrays, one can measure expression patterns of large numbers of genes in different cell types (such as cancer cells versus normal cells, or liver cells versus kidney cells). It can also be used to examine the changes in gene expression over time (for example, as an embryo develops), or changes in a given cell type under different environmental conditions (various temperatures, for instance).

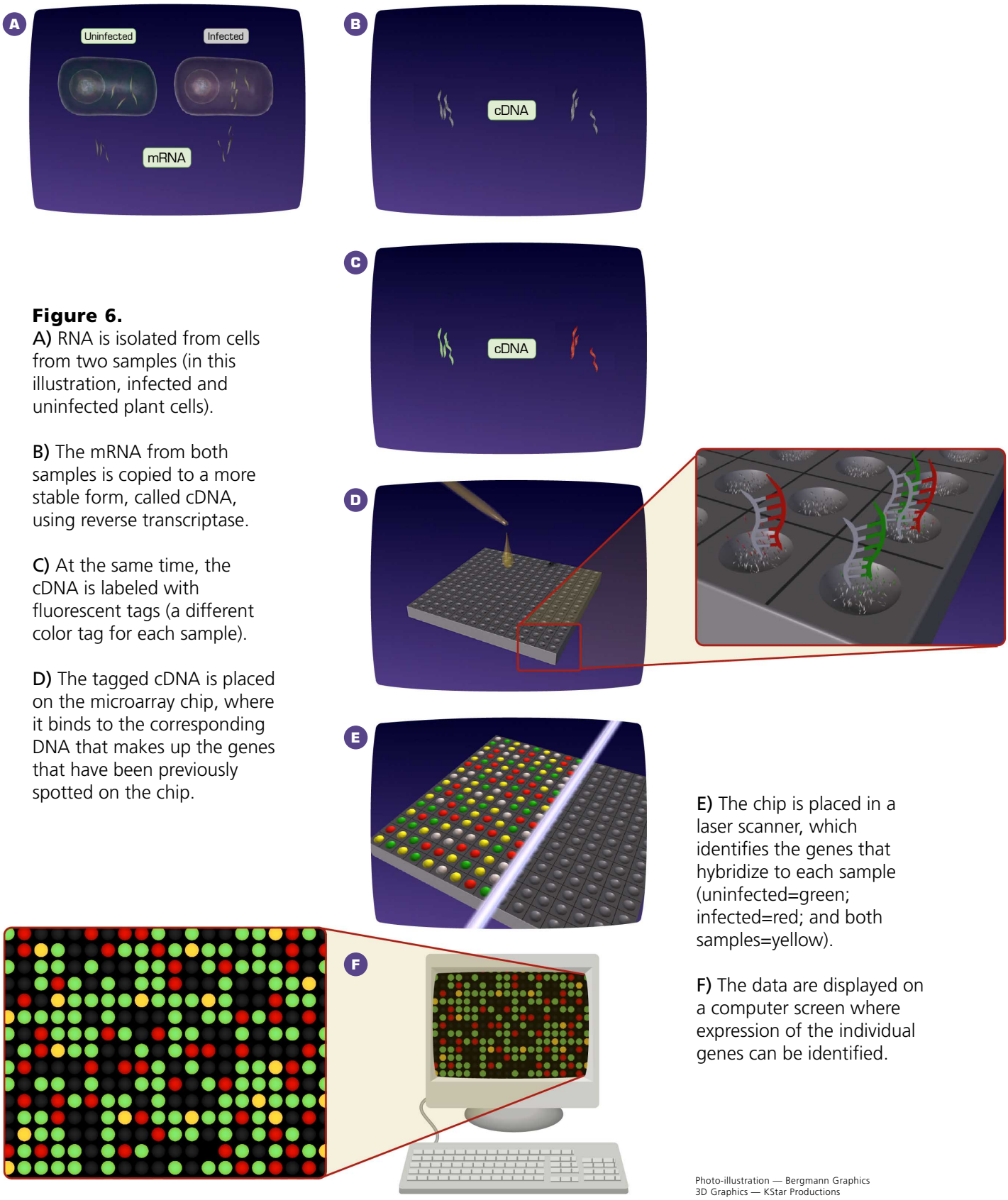


Figure 6.
A) RNA is isolated from cells from two samples (in this illustration, infected and uninfected plant cells).

B) The mRNA from both samples is copied to a more stable form, called cDNA, using reverse transcriptase.

C) At the same time, the cDNA is labeled with fluorescent tags (a different color tag for each sample).

D) The tagged cDNA is placed on the microarray chip, where it binds to the corresponding DNA that makes up the genes that have been previously spotted on the chip.

E) The chip is placed in a laser scanner, which identifies the genes that hybridize to each sample (uninfected=green; infected=red; and both samples=yellow).

F) The data are displayed on a computer screen where expression of the individual genes can be identified.

Photo-illustration — Bergmann Graphics
3D Graphics — KStar Productions

Ethics

Possessing detailed knowledge about the genetic makeup of individuals raises several complex ethical quandaries. How confidential should genetic information be? How should privacy concerns be weighed against other interests? If genetic information related to disease genes should be as confidential as any other health-related information, should there be databases of detailed genomic information on individuals? Even without detailed genomic databases, thirteen genetic markers are sufficient for the FBI to identify every person except identical twins. Should this type of genetic information be stored on all convicted criminals; everyone arrested for a crime; or on every individual, regardless of his or her past? Who should have access to detailed genetic information if it becomes available? Should it be accessible to law enforcement officers, physicians, research scientists, employers and potential employers, or insurance providers?

Sir Alec Jeffreys, the scientist who first developed the technique of genetic fingerprinting in Great Britain, is a proponent of a DNA database that contains the genetic profile of every individual in that country. To provide anonymity, however, he suggests that the actual identity of each individual be kept in a separate database with high security. Only certain circumstances, such as a link to a crime, would justify identification of the individual.

The NIH-DOE Working Group on the Ethical, Legal, and Social Implications (ELSI) has recommended that employers can request and use genetic information, but only to protect the health and safety of workers; such information must remain confidential. They also recommend that insurers cannot use genetic information to deny or limit health insurance coverage or to charge different fees based on this information. Overall, the focus of legislation should be to prevent discrimination of individuals based on genetic information.

In 1993, long before the human genome was completed, a committee of the Institute of Medicine of the National Academy of Sciences developed recommendations to prevent involuntary genetic testing and protect confidentiality. They concluded that the responsible use of genetic testing requires that individuals understand the tests, their significance, and their implications. Testing for diseases should be done only when individuals are capable of providing informed consent. This means not only that individuals must be informed, but that they also should understand the implications of that consent. Such informed consent requires an understanding of genetics by the public. Education in genetics must be increased to ensure that future generations have this knowledge.

Patenting of human genes is another ethical concern emerging from the human genome project. In order to be patentable under the U.S. Federal Patent Act, an invention must be "novel, nonobvious, and have utility." In applying for a patent on a human gene, applicants generally claim that the patent's holders will add to the utility of the natural gene by developing tests and therapies to fight diseases associated with that gene. Opponents of gene patenting think that patents will limit the ability of other scientists to do additional research on these genes.

Most patents are filed by private companies that plan to develop and market diagnostic tests and treatments that come from their research on a particular gene. These companies feel that, without a patent, they cannot afford to do the research that will lead to useful products. They argue they need the protection of a patent before they can invest millions of dollars in the development of new tests, drugs, and therapies. Some scientists counter that companies tend to patent genes even before they know what the gene does, so it is hard to understand how they can claim that they will increase the utility of such a gene. Making scientific data freely available, while still protecting the interests of private organizations that will provide the practical uses for the data, would be in the best interest of everyone.

Epilogue

The explosion of information coming from the sequencing of genomes has changed the landscape of biology. We now have tools to better understand the basis of disease and its prevention and control. These tools also allow us to design, more effective drugs, and even understand the genetic relationships among all living things that make the universal tree of life. Acquiring the sequence was only the beginning.

References

1) Venter, J. C. 1998. Testimony before the subcommittee on energy and environment. U. S. House of Representatives Committee on Science. 17 June 1998.

2) Dulbecco, R. 1986. A turning point in cancer research: Sequencing the genome. *Science* 231:1055–1056.

Further Reading

Book

Krane, D. E., and Raymer, M. L. 2003 *Fundamental concepts of bioinformatics.* San Francisco: Benjamin/Cummings.

Articles

Adams, A. 2002. Prospecting for gold in genome gulch. *The Scientist* 16:36–38.
Modern-day bioprospectors combine association, functional, and gene expression data to stake their claims in the rich veins of human DNA.

Ezzell, C. 2003. *Scientific American: Beyond the human genome.*
An e-book that describes the challenges remaining now that we have sequenced the human genome.

Friend, S. H., and R. B. Stoughton. 2002. The magic of microarrays. *Scientific American* 286:44–53.
DNA microarrays could hasten the day when custom-tailored treatment plans replace a one-size-fits-all approach to medicine.

Howard, K. 2000. The bioinformatics gold rush. *Scientific American* 283:58–63.
A $300-million industry has emerged around turning raw genome data into knowledge for making new drugs.

Kling, J. 2002. Speed-reading the genome. *The Scientist* 16:49.
With a novel approach, US Genomics shoots for viable, real-time genomic sequencing.

Klotzko, A. J. 2000. SNPs of disease. *Scientific American* 282:28.
The U.K. plans a national genomic database to study late-onset sickness.

Pistoi, S. 2002. Facing your genetic destiny, Part II. *Scientific American* Explore Online.
Finding treatments that match individual gene profiles is the next frontier in drug research and the objective of a new science called pharmacogenomics.

Stix, G. 2002. Legal circumvention. *Scientific American* 287:36.
Molecular switches provide a route around existing gene patents.

Glossary

BAC. Bacterial artificial chromosome. A plasmid vector used to clone large fragments of DNA (average size of 150 kb) in *E. coli*.

BLAST. Basic local alignment search tool. A computer program that identifies homologous (similar) genes in different organisms.

Clone-based sequencing. A genomic sequencing strategy that is based on a hierarchical approach. It uses mapping, cloning of large DNA fragments, and small DNA fragments in plasmids to organize the sequenced fragments of DNA into a single complete sequence.

cDNA. Also known as complementary DNA. DNA produced by reverse transcribing mRNA. It has the same sequence as the mRNA (except that a U is replaced by a T).

CLUSTAL. A computer program that aligns conserved regions in multiple DNA or protein sequences. Used to determine the evolutionary relationships among genes or proteins.

DNA fingerprint (DNA profile). Nucleotide sequence variants that are characteristic of an individual and can be used as a unique identifier of that individual.

Exon. The sequence of a gene that encodes a protein. Exons may be separated by introns.

Haplotype. Particular patterns of SNPs on a chromosome that are inherited together as a block.

Homologous (homology). Similarity of genes or other features of organisms due to shared ancestry.

Intron. The DNA sequence within a gene that interrupts the protein-coding sequence of a gene. It is transcribed into RNA but it is removed before the RNA is translated into protein.

Knockout study. Inactivation of a specific gene; typically used in laboratory organisms to help to determine gene function.

Microarray chip. Set of miniaturized biochemical reactions that occur in small spots on a microscope slide that may be used to test DNA fragments, antibodies, or proteins.

Open reading frame (ORF). The DNA or RNA sequence between the start codon sequence and the stop codon sequence.

Plasmid. A small, circular, self-replicating, extrachromosomal piece of DNA. Many artificially constructed plasmids are used as cloning vectors.

Polymorphism. The presence of two or more variants of a genetic trait in a population.

Protein motif. A pattern of amino acids that is conserved across many proteins and c onfers a particular function on the protein.

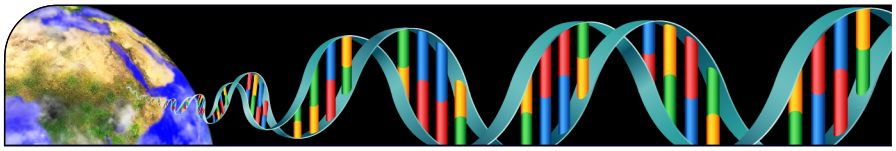
Short tandem repeat (STR). Multiple adjacent copies of an identical DNA sequence in a particular region of a chromosome.

Single nucleotide polymorphism (SNP). Variations in the DNA sequence that occur when a single nucleotide (A, T, C, or G) in the genome sequence is changed.

Synonymous mutation (silent mutation). A change in a nucleotide in the DNA sequence that does not result in a change in the amino acid in the protein.

Transposable element. A type of DNA that can move from one chromosomal location to another.

Whole genome shotgun sequencing. A genomic sequencing strategy that is based on cloning and sequencing millions of very small fragments of DNA, and then using computer programs to align the sequences together.



REDISCOVERING BIOLOGY

Molecular to Global Perspectives

Proteins and Proteomics

"If DNA is the genetic blueprint then what is the proteome? What are the proteins of the cell? The proteins of the cell are the walls, the floor, the plumbing, the beds, the furniture, the sinks, the glasses — everything that goes on in the house. All of those processes are being carried out by proteins and so DNA may be providing the instructions but all the work is really being done by the proteins." STANLEY FIELDS, PHD

What Is Proteomics?

A bacterial cell may seem simple but it's actually a complex structure — a gel-like matrix of the cytoplasm, surrounded by both a lipid bilayer cell membrane and a cell wall. The cell must perform many functions including the intake of nutrients, the metabolism of those nutrients, growth, cell division, and the excretion of wastes. What molecules are involved? Although the cytoplasm contains water, proteins, carbohydrates, various ions and assorted other molecules, proteins do most of the work. A typical bacterium requires more than 4,000 proteins for growth and reproduction. Not all of the proteins are made at the same time and some are made only under special conditions, such as when the cell is stressed or finds itself in a novel environment. The complement of proteins found in this single cell in a particular environment is the **proteome**. Proteomics is the study of the composition, structure, function, and interactions of the proteins directing the activities of each living cell.

If a bacterial cell needs more than 4,000 proteins, how many can we expect to find in animals? Mammals, including humans, have probably more than 100,000 proteins. Although the genome contains the genetic blueprint for an organism, the proteins of eukaryotes provide the unique structure and function that defines a particular cell or a tissue type, and ultimately defines an organism. Different types of cells make different proteins, so the proteome of one cell will be different from the proteome of another. In addition, cells that result from a disease, such as cancer, have a different proteome than normal cells. Therefore, understanding the normal proteome of a cell is critical in understanding the changes that occur as a result of disease. This knowledge can lead to an understanding of the molecular basis for the disease, which can then be used to develop treatment strategies. Knowing how the proteome changes as the organism grows may also provide insight into the mechanisms of development in healthy organisms.

Under the classical concept of "one gene makes one enzyme," the proteome would simply comprise the products of all the genes present in the genome of an organism. But it is not that simple. The number of genes identified in the human genome is only about 30,000–35,000. How can only 35,000 genes encode more than 100,000 proteins? There are several possible answers to this question, which will be discussed in more detail below. One answer is that each gene may encode several proteins in a process called alternative splicing. **Alternative splicing** means that one gene may make different mRNA products and, hence, different proteins. Another answer is that one protein may be modified chemically after it is synthesized so that it acquires a different function. A third answer is that proteins interact with each other in complex pathways and networks of pathways, which may change their function. So, one gene may produce several, functionally different proteins in a variety of ways.

Introduction to Protein Structure

Although proteins are unique, they share certain common characteristics (**Fig. 1**). The **primary structure** of each protein is determined by the sequence of specific amino acids, encoded by the mRNA, which directs the proper folding of the polypeptide chain into the secondary structure. One type of **secondary structure** is the alpha helix, a region of the polypeptide that folds into a corkscrew shape. Beta strands are linear structures of polypeptides, bonding together to form a flat beta sheet. Other regions of secondary structure may include turns and random coils. These helices, strands, turns, and coils interact chemically with each other to form the unique three-dimensional shape of the protein, called the **tertiary structure**. For some proteins, a single polypeptide chain folded in its proper three-dimensional structure creates the final protein. Many proteins, however, have several different polypeptide subunits that make the final active protein. For these proteins, the interactions between the different subunits form the **quaternary structure**.

Discrete portions of proteins can fold independently from the rest of the protein and have their own function. These are called **domains**, and serve as one of the building blocks of that protein. Domains are evolutionarily mobile, capable of rearranging as new proteins evolve. There are thousands of structural domains, and many of them have been conserved widely across proteins. New proteins appear to have arisen over evolutionary time by bringing different domains together in a process known as **domain shuffling**. Domains often contain smaller **motifs**, consisting of a conserved pattern of amino acids, or of combinations of structural elements formed by the folding of nearby amino acid sequences. An example of a motif is a helix-loop-helix, which binds to DNA. Very similar motifs are found in many proteins that are not related. Scientists have classified conserved domains and motifs in a number of databases so that new proteins can be easily analyzed for the presence of these elements.

Determining Protein Structure

While determining the polypeptide sequence resulting from gene translation is straightforward, determining the actual three-dimensional (3D) structure requires some sophisticated experimental techniques. One such long-standing technique is **X-ray crystallography**, which is

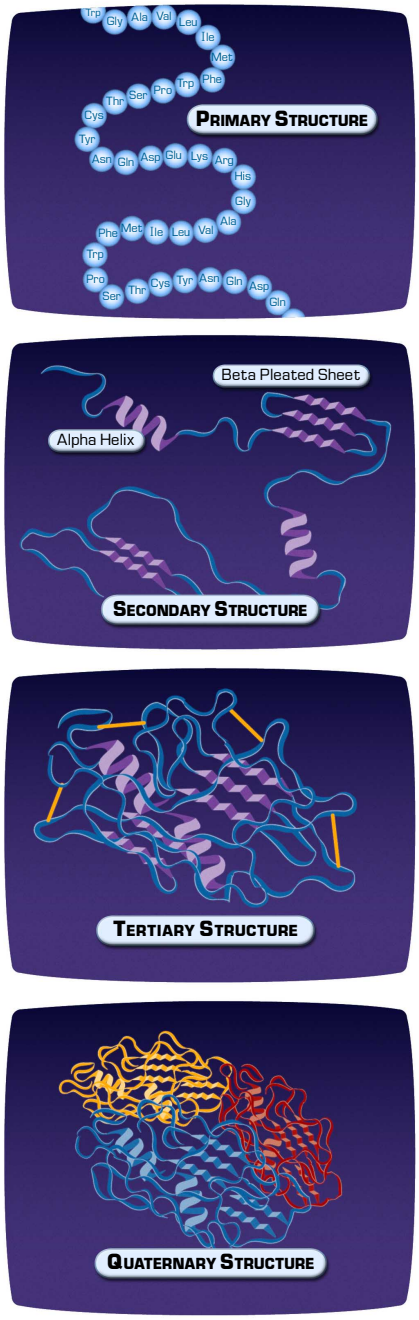


Photo-illustration — Bergmann Graphics

Figure 1. Primary Structure: The specific sequence of amino acids in a polypeptide chain. Secondary Structure: The folding of the polypeptide chain into specific shapes, such as the alpha helix and beta pleated sheet. Other regions of secondary structure may include turns and random coils. Tertiary Structure: The unique three-dimensional shape that is the result of chemical interactions between amino acids that fold the regions of secondary structure. Quaternary Structure: The specific interaction of two or more polypeptide subunits.

based on the scattering of X-rays by the electrons in the crystalís atoms. Think of the regular structure of table salt crystals. The atoms forming that structure are spaced very precisely in the crystal. Due to this regular spacing, a particular diffraction pattern forms when X-rays strike it. One can reconstruct the position of each atom in the crystal by observing the diffraction pattern and, thus, can make a three-dimensional map of the molecule. Although proteins are much more complex than table salt, researchers have crystallized many of them in their native configuration and have used X-ray crystallography to find their 3D structures. The 3D structures of proteins are available to all scientists in a public database called the "Protein Data Bank."

Not all proteins can be crystallized, however. For example, membrane proteins have many hydrophobic amino acids and are particularly difficult to crystallize. A different technique to analyze proteins in solution is **nuclear magnetic resonance (NMR)**. NMR is based on the principle that the nuclei of some elements' atoms, such as hydrogen, resonate when a molecule, such as protein, is placed in a powerful magnetic field. NMR measures chemical shifts of the atoms' nuclei in the protein, which is dependent on nearby atoms and on their distances from each other. The signals that NMR produces are a set of distances between specific pairs of atoms. NMR data generate models of possible structures, rather than a single structure. For smaller proteins in particular, NMR can quite accurately predict the 3D structure.

Despite advances in techniques for determining protein structure, the structures of many proteins are still unknown. With the help of protein prediction programs, computer analysis of genome sequences is producing thousands of new *hypothetical* proteins of unknown structure and function. These proteins are called "hypothetical proteins" because they represent the products predicted from the gene sequence; however, there is, as yet, no evidence that they are actually made and there is no known function for them.

Computer programs may help determine the structure of proteins whose function is not yet known. By comparing the sequence of the unknown protein to proteins with known 3D structures, these programs can make a predictive model of the unknown protein's structure using the known proteins as templates. The success of this method depends on the quality of the match between the known template proteins and the unknown target protein. In addition, when the function of the template protein is known, it may help identify the function of the unknown protein. These prediction programs do not produce structures with the detail or reliability of experimental techniques such as X-ray crystallography. They do, however, provide a means to analyze — in a reasonable time period — the large number of new proteins identified by the analysis of whole genomes.

Structure and Function Relationships of Proteins

The three-dimensional structure of a protein defines not only its size and shape, but also its function. One characteristic that affects function is the hydrophobicity of a protein, which is determined by the primary and secondary structure. For example, let's look at membrane proteins. Membranes contain large amounts of lipids, which are

notoriously hydrophobic (water and oil donít mix). The membrane-spanning regions of membrane proteins are typically alpha helices, made of hydrophobic amino acids. These hydrophobic regions interact favorably with the hydrophobic lipids in the membrane, forming stable membrane structures.

Hemoglobin is a soluble protein — found in the cytoplasm of red blood cells as single molecules — which bind oxygen and carry it to the tissues. In sickle cell anemia, a mutation in the beta-globin protein of the red blood cell increases its hydrophobicity and causes the mutant protein molecules to stick to each other, avoiding the aqueous environment. Chains of hemoglobin change the shape of the red blood cell from round to a sickle shape, which causes the cells to collect in narrow blood vessels.

The folding of a protein allows for interactions between amino acids that may be distant from each other in the primary sequence of the protein. In enzymes, some of these amino acids form a site in the structure that catalyzes the enzymatic reaction. This site, called the **active site** of the enzyme, has amino acids that bind specifically to the substrate molecule, also called a **ligand (Fig. 2)**. In a similar manner, certain sites in cell receptor proteins bind to specific ligand molecules that the receptor recognizes.

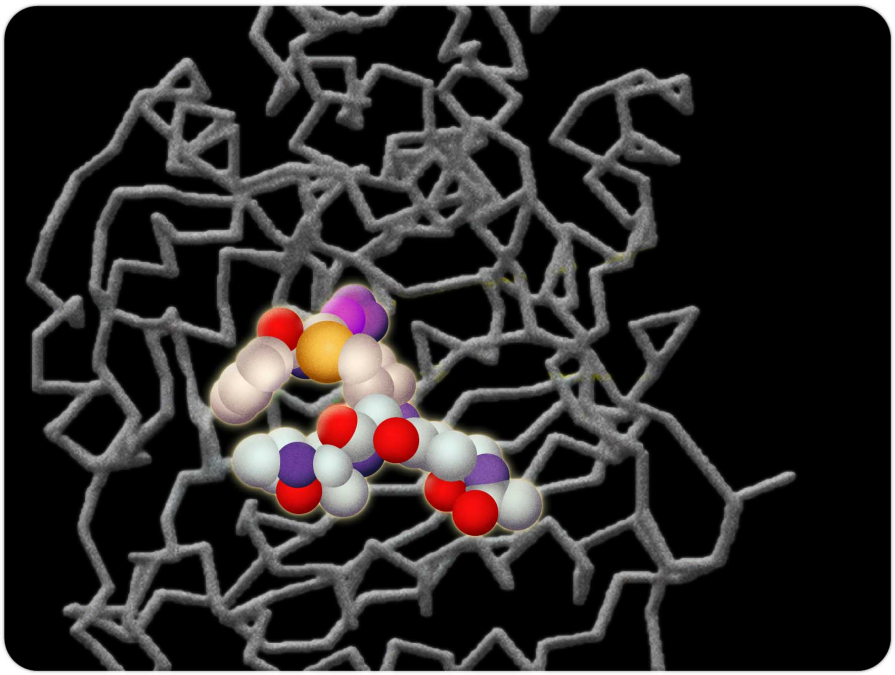


Photo-illustration adaption — Bergmann Graphics

Figure 2. The active site of the penicillin-binding protein. The gray stick-like structures represent the secondary and tertiary structure of the penicillin-binding protein. Binding of the antibiotic, the substrate, to the active site blocks the normal action of the protein in the bacterial cell, resulting in death of the cell.

Alterations in amino acids that may be distant from each other in the primary sequence can lead to changes in folding. It may also cause changes in chemical interactions among amino acids at the active site, which alter the enzyme activity or binding of the ligands to receptor proteins. Binding of ligands to an active site requires specific amino acids. Therefore, an active site in a new enzyme that belongs to the same family as a known enzyme can usually be identified by its similarity to the active site of the known protein. Computer programs can use the information from a database of known enzymes to predict the active site of a new protein using a template-based method, similar

to that described above for determining the three-dimensional structure of a protein. Once the program has identified the potential ligand-binding sites, other programs can test the fit and the binding ability of thousands of possible ligand molecules — even theoretical ligands that may not yet exist. This has tremendous possibilities for the design of new drugs, particularly for cancer therapy.

Protein Modification

The complexities of the 3D structure of proteins are not the only difficulty in characterizing proteins. Many proteins contain additional chemicals that modify their structure. The final structure of a protein may include any number of modifications that occur during and after the synthesis of the protein on the ribosome. These post-translational modifications change the size and the structure of the final protein. Some modifications occur after a protein is made; others occur during translation of the protein, and are required for proper folding of the protein. One possible modification is enzymatic cleavage of the original polypeptide by proteases to produce a smaller product. Other modifications include the addition of sugar molecules to certain amino acids in the protein (**glycosylation**), or the addition of a phosphate group (**phosphorylation**) or a sulfate group (sulfation).

Many proteins are modified by proteases that remove short peptides from either end of the protein. The shortened polypeptides then fold into an active protein. One of the most common of these cleavages is the removal of specific signal peptides. These peptides target proteins for transport to a particular cellular organelle in a process known as **protein sorting**. An example of this is the hormone insulin, which is made as preproinsulin. After removal of the 24-amino-acid signal peptide from preproinsulin to form proinsulin, the latter polypeptide is further processed in the endoplasmic reticulum. This produces the final hormone, insulin, which is released from the cell.

Glycosylation — the addition of specific short-chain sugars to asparagine, serine, or threonine — is very common in membrane proteins that form structural components of the cell surface. These proteins, called glycoproteins, are important in many cell processes, including binding by receptors and eliciting an immune response. Glycoproteins are often specific cell markers. For example, ABO blood types result from the presence or absence of specific glycoproteins (A-type, B-type, both, or neither) on the surface of red blood cells. Human immunoglobulin G (IgG) is also a glycoprotein in which the sugar appears to very important for the normal function of the protein in the immune response. Scientists have discovered that abnormal sugars in IgG strongly correlate with the autoimmune disease called rheumatoid arthritis, characterized by chronic joint inflammation, and the presence of antibodies directed against IgG and other host proteins.

Reversible phosphorylation of threonine, serine, or tyrosine residues by enzymes called **kinases** (which add a phosphate) and **phosphatases** (which remove the phosphate) play an important role in the regulation of many cell processes, such as growth and cell cycle control. (See the *Cancer* unit.) Phosphorylation may occur sequentially from one protein to another, resulting in a series of activations called a "phosphorylation cascade."

Genomics-Based Predictions of Cellular Proteins

We now have large databases of gene sequences, predicted protein sequences, and known 3D protein structures; yet we still don't know the total protein composition of a cell. Determining the proteome of a cell is a complicated task. There are two approaches to obtaining this information: computer-based and experimental.

The computer-based method uses the genome sequence of an organism to predict genes, based on known characteristics of protein-coding regions of the genome. (See the *Genomics* unit for a discussion of computer-based methods for gene identification and microarrays to identify expressed genes.) However, even if we know that a particular sequence is a gene, we don't necessarily know all the possible proteins it makes.

One reason is that one gene may produce more than one mRNA. RNA splicing is the normal process in which **intron** sequences are removed from the pre-mRNA, producing the mRNA, which corresponds to the **exons**. However, some transcripts can be spliced in alternative ways (alternative splicing), joining different exons (**Fig. 3**). The result is two or more different mRNA molecules from one gene. Variants of a protein produced by alternative splicing may have a similar physiological activity, a different and unrelated activity, or no activity at all. According to one estimate, about forty percent of human genes are alternatively spliced. This is one mechanism that accounts for the relatively large number of proteins produced by only about 35,000 human genes.

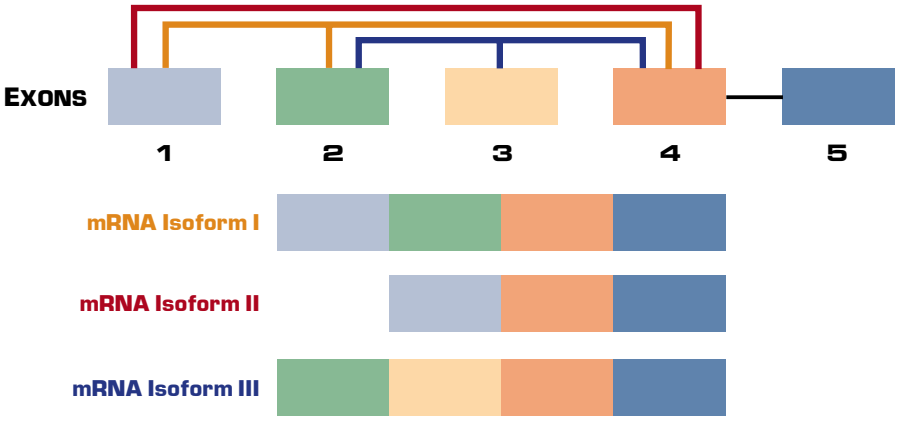


Figure 3. More than one protein can be made from a gene. In this case, three different mRNA molecules are made from one gene. The exons (the numbered boxes) can combine in different configurations to eventually form different proteins.

A more direct approach to identify proteins in a cell is to measure enzyme activities and other functions for which there are biochemical assays. In some cases, we can identify the function of new proteins by combining our knowledge of metabolic pathways in many organisms with the predicted function from genome analysis. With this type of information, researchers can readily identify new enzymes. To do this, they examine the similarity of the genome sequences to known enzymes, as well as the presence (in the same genome) of the proteins that are required for the other steps in the metabolic pathway.

2D Gel Electrophoresis to Identify Cellular Proteins

While computer-based methods are powerful, they can only predict the function of proteins for which some information is already available. How do we understand the proteins that we don't already know about? This requires experimental approaches.

One way to identify proteins is to extract all the proteins from a sample of cells and separate them in a gel matrix, using a technique called **polyacrylamide gel electrophoresis** (PAGE). The proteins are separated by size, with the smaller proteins moving faster through the gel than the larger proteins. After staining, a pattern of bands appears that corresponds to the proteins in the cell. However, this technique can only resolve a few hundred proteins, and cannot separate proteins of very similar size.

A modification of this procedure — called **2D gel electrophoresis** — separates proteins into two dimensions, using two different characteristics. Proteins are separated in the first dimension by their **isoelectric point (pI)**, the specific point at which the net charge of the protein is zero. These separated proteins, in a flat gel strip, are then placed on a standard polyacrylamide gel. Every protein band that was separated in the first dimension according to its isoelectric point is now separated in the second dimension by its size. The result is small spots, each representing a protein; even proteins of the same size will be resolved if they have a different isoelectric point. A good 2D gel can resolve one thousand to two thousand proteins, which appear, after staining, as dots in the gel (**Fig. 4**). This technique is useful when comparing two similar samples to find specific protein differences; for example, comparing the proteins in a tumor cell versus a normal cell. However, it can miss very small proteins or non-abundant proteins.

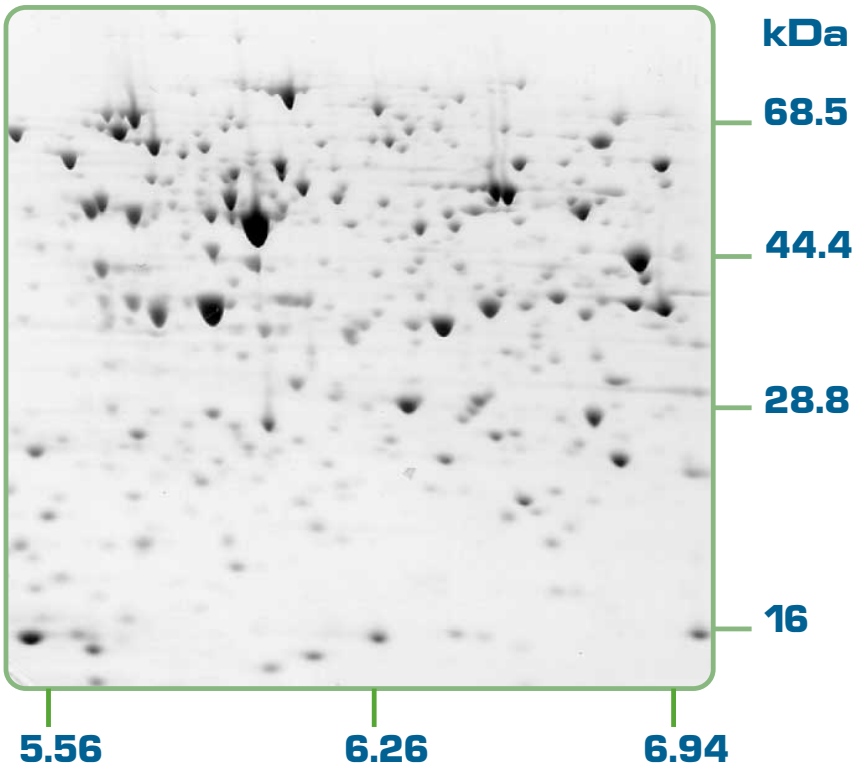


Figure 4. *Haemophilus influenzae* cell proteins separated by 2D gel electrophoresis. The basic proteins are to the right of the gel and the acidic proteins to the left. High molecular weight proteins are to the top of the gel.

Phil Cash, PhD, 2D GEL.
Courtesy of Phil Cash, PhD, University of Aberdeen.

Mass Spectrometry to Identify Cellular Proteins

While the 2D gel method easily separates proteins, it doesn't identify them. If there are differences in spots between the proteins in a cancer cell and a normal cell, this method cannot determine the actual identity of the different proteins in the two cell types. To identify these proteins, individual spots are excised from 2D gels and then subjected to **mass spectrometry**, which separates charged particles, or ions, according to mass. First the molecules in the sample are ionized to produce a population of charged molecules. A mass analyzer then separates the sample's molecules based on their mass to charge ratio. A detector then produces a peak for each ion; this peak gives the mass and represents the amount of the ion. A computer program reads the complex spectral information from the mass spectrometry process. The program matches the information on the each peptide's mass against the mass of theoretical, predicted peptides, based on known proteins in databases. This is called **peptide mass mapping**. With many different peptides for each protein, the computer can match the sequence to one or more known proteins. Peptide mass mapping can only be used in situations where the genome has been sequenced and all predicted proteins for the genome are known.

Another application of mass spectrometry is **protein fingerprinting**. This technique has been used to identify unique sets of proteins in blood, which serve as markers for different forms of cancer. Interestingly, for this method to be useful, we do not need to know the actual identities of the particular proteins used as markers for a disease. Instead, this technique relies on pattern recognition software. Using training data from samples from individuals with and without cancer, the program searches for a particular pattern of peaks that correlates with cancer. This technique requires only a drop of blood and does not require any detailed genetic information; however, its accuracy in predicting some forms of cancer is limited because the number of marker peptides is not sufficiently large. As more samples are evaluated, the accuracy will likely increase because the software will be able to find more accurate peptide patterns correlating to cancer. Proteomic fingerprinting holds great promise as a diagnostic tool for a variety of diseases that produce distinctive patterns of proteins in blood.

Identifying Protein Interactions

While it is convenient to think of proteins as discrete and independent molecules, this is actually an oversimplified view. Many proteins require other proteins or cofactors for activity; and proteins involved in signal transduction, protein trafficking, cell cycle, and gene regulation must interact with other proteins in those processes. Many of these interactions require particular domains called **interaction domains**. Proteins involved in the interactions contain combinations of interaction domains (for interaction with other proteins) and **catalytic domains** (for function of the protein). The interaction domain can bind the partner protein, even in the absence of the rest of the protein. Interaction domains are often quite versatile, capable of binding a variety of related ligands. In addition, one protein may contain several different interaction domains. The modular nature of

these domains allows the protein to interact with multiple target proteins in the cell; thus it provides a mechanism for integration and control of information from protein to protein in a cell. Such protein-protein interactions form the basis for our current understanding of cell signaling pathways and protein networks that regulate all the activities in a cell.

Because protein-protein interactions regulate the activities of cells, identifying them is critical to understanding cellular processes. Mass spectrometry techniques have been developed for large-scale screening to identify interacting proteins. For example, hundreds of known proteins in yeast were engineered to contain a biochemical tag that would allow the tagged protein to be separated from other proteins in a cell extract. This was done gently so that other proteins bound to the tagged protein would still be attached. The tagged protein, along with any associated proteins, was then analyzed by mass spectrometry. The results revealed that about eighty-five percent of these proteins were associated with other proteins. Although most interacted with many other proteins, in some cases two different protein complexes had at least one protein in common. Among the most intriguing questions to come out of this research were what controls which proteins interact and — for those that interact in multiple complexes — how do these proteins know which complex to join?

The Yeast Two-Hybrid System

The **yeast two-hybrid system** is a powerful technique for identifying multiprotein complexes. Using genetically engineered yeast, scientists can identify complexes when specific pairs of interacting proteins activate expression of a reporter gene. One often-used reporter gene is the lacZ gene. When two proteins interact in the yeast cell they activate expression of this gene, allowing yeast cells to metabolize an indicator that turns these cells a different color. The interacting proteins are then identified from the colonies formed by these colored cells. The two-hybrid system has been expanded to use microarrays of cloned yeast genes (see below). These large-scale yeast two-hybrid assays can provide information on thousands of protein-protein interactions. Using this technology, researchers are identifying all the proteins in yeast that interact, and they will then map the complex network of cellular functions to these interacting proteins.

Protein Microarrays

Another strategy for the large-scale study of proteins is similar to the DNA microarrays, which measure gene expression in different cells types. (See the *Genomics* unit.) Based on the rapid, large-scale technology (often called **high-throughput technology**) that was developed for DNA microarrays, scientists have developed similar microarrays for proteins. In a protein microarray, very small amounts of different purified proteins are placed on a glass slide in a pattern of columns and rows. These proteins must be pure, fairly concentrated, and folded in their active state. Various types of probe molecules may be added to the array and assayed for ability to bind or react with the protein. Typically the probe molecules are labeled with a fluorescent dye, so that when the probe binds to the protein it results in a fluorescent signal that can be read by a laser scanner.

This technology can complement other techniques, such as mass spectrometry and yeast two-hybrid assays, to identify thousands of protein-protein interactions. Protein arrays can be screened for their ability to bind other proteins in a complex, receptors, antibodies, lipids, enzymes, peptides, hormones, specific DNA sequences, or small molecules, such as potential new drugs. One of the most promising applications for protein microarrays is the rapid detection or diagnosis of disease by identifying a set of proteins associated with the disease.

One example of the use of this technique is the development of a microarray that may help in the treatment of cancer. This microarray contains many different mutant forms of a protein called p53. P53 is an anti-cancer protein, called a "tumor-suppressor protein," and about half of all cancers have mutations in p53. (See the *Cancer* unit.) Researchers can screen the immobilized mutant p53 proteins in the microarray for biological activity, as well as for new drugs that can restore its normal tumor-suppressing function.

Protein Networks

The cell is a complex and dynamic system of networks of interacting molecules. An understanding of the cell requires analyzing these complex interactions as a system. Systems biology takes the approach that the powerful high-throughput techniques, developed as part of whole genome and proteome analysis, will allow the simultaneous study of complex interactions of networks of molecules, including DNA, RNA, and proteins. Fully understanding complex networks of molecular interactions in the cell requires a combination of several different experimental techniques, including DNA and protein microarrays, mass spectral analysis, and two-hybrid analysis. This, combined with the power of computers to analyze the massive amount of data, produces models of interacting networks, which better describe the workings of a cell (**Fig. 5**).

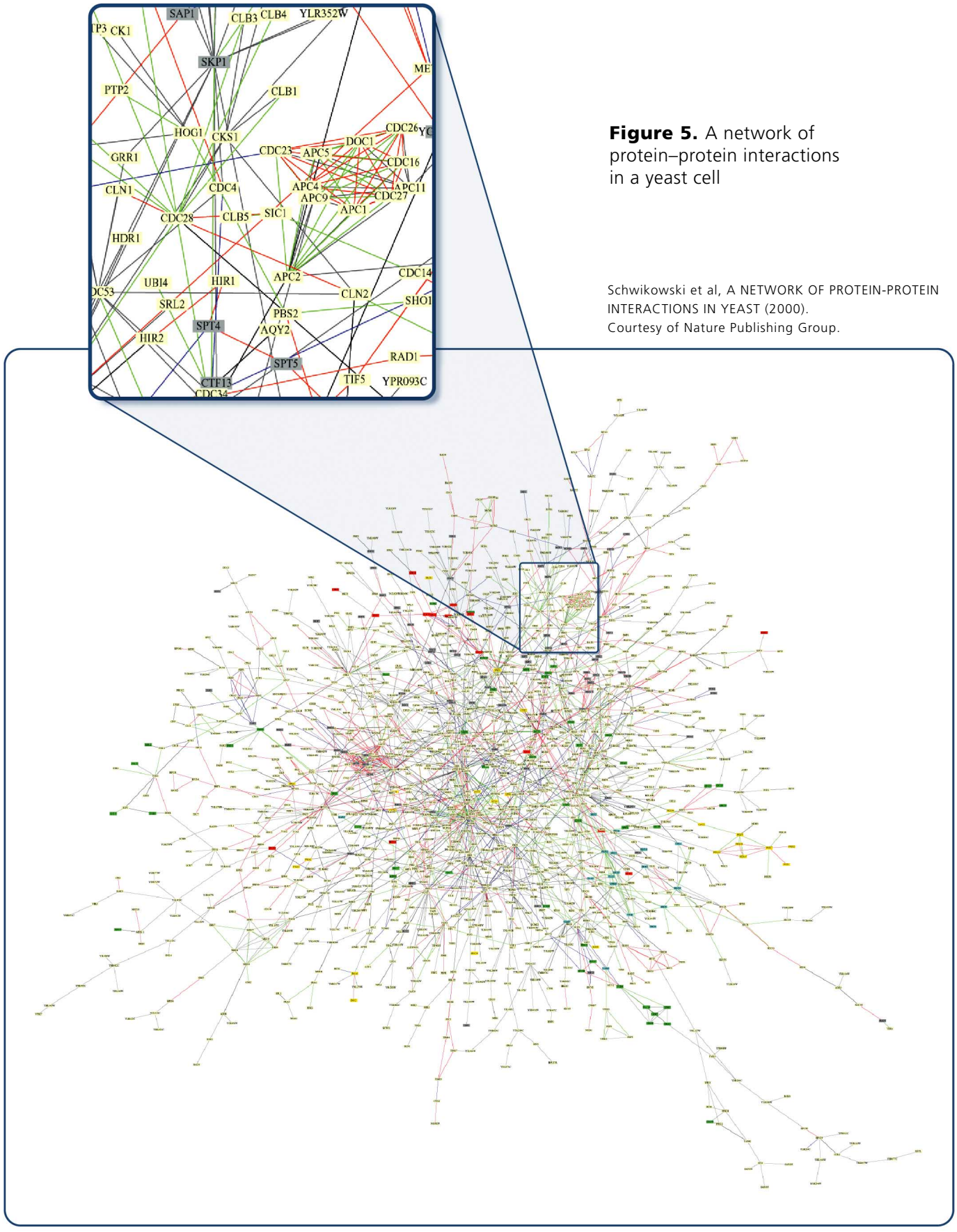


Figure 5. A network of protein–protein interactions in a yeast cell

Schwikowski et al, A NETWORK OF PROTEIN-PROTEIN INTERACTIONS IN YEAST (2000). Courtesy of Nature Publishing Group.

Proteomes in Different Organisms

Although scientists have sequenced dozens of genomes from organisms as diverse as viruses, bacteria, nematode, fruit fly, puffer fish, mouse, and human, we still don't know what uniquely characterizes each of these organisms. For example, both mouse and human genomes contain around 30,000 genes. How many of these genes do they share? Based on comparisons of the two genomes, ninety-nine percent of the genes are conserved in both species and are, thus, derived from a common evolutionary ancestor. The remaining one percent represents genes that evolved independently in mouse or human. If these two organisms share so many similar genes, how can they be so different? A simple example may help us to understand that the presence of a gene does not mean that the protein is expressed. Pigs produce cell surface proteins, which are modified by glycosylation to contain a sugar called galactose (GAL). Those GAL-proteins, present in pig blood vessels, are seen as foreign by the human immune system. This leads to the very rapid destruction of pig organs that have been transplanted into humans when a human organ was not available. Interestingly, humans lack GAL-proteins but still have the gene for making them; the gene is not expressed in humans. Therefore, the presence of a gene does not mean that it is expressed. In fact, every somatic cell in an organism shares the same genes; so, the differences between tissue types — say liver and heart — result from differences in gene *expression*. (See the *Genes and Development* unit.)

Identification of proteins may provide the most useful information in determining the significant differences between species. How different are the proteins in even closely related organisms? With the development of proteomic techniques, scientists are beginning to tackle this difficult question. One answer is that very similar genes in two organisms may be expressed very differently. Dr. Svante Pääbo of the Max Planck Institute for Evolutionary Anthropology analyzed the proteins from brains of human and chimps. (See the *Genomics* and *Human Evolution* units.) He found that many very similar genes produced much more protein in human brain cells than in chimp brain cells. In contrast, the same type of experiment done with blood or liver cells showed much less difference between human and chimp in the amount of protein produced.

At a different level, there are some clear differences in protein composition between the cells of eukaryotes and those of the other kingdoms. One is that eukaryotes have many more long proteins, more proteins with regular secondary structure and less random globular structure, and more loop regions in their proteins. Certain conserved structural domains show up in proteins, but are used in a number of different pathways. While there are many protein homologues conserved across many different organisms, some proteins are unique to one organism. As more genomes and proteomes are characterized, comparative genomics and proteomics will allow scientists to further understand how organisms differ.

Proteomics and Drug Discovery

One of the most promising developments to come from the study of human genes and proteins has been the identification of potential new drugs for the treatment of disease. This relies on genome and

proteome information to identify proteins associated with a disease, which computer software can then use as targets for new drugs. For example, if a certain protein is implicated in a disease, the 3D structure of that protein provides the information a computer programs needs to design drugs to interfere with the action of the protein. A molecule that fits the active site of an enzyme, but cannot be released by the enzyme, will inactivate the enzyme. This is the basis of new drug-discovery tools, which aim to find new drugs to inactivate proteins involved in disease. As genetic differences among individuals are found, researchers will use these same techniques to develop personalized drugs that are more effective for the individual.

Virtual ligand screening is a computer technique that attempts to fit millions of small molecules to the three-dimensional structure of a protein. The computer rates the quality of the fit to various sites in the protein, with the goal of either enhancing or disabling the function of the protein, depending on its function in the cell. A good example of this is the identification of new drugs to target and inactivate the HIV-1 protease. The HIV-1 protease is an enzyme that cleaves a very large HIV protein into smaller, functional proteins. The virus cannot survive without this enzyme; therefore, it is one of the most effective protein targets for killing HIV (**Fig. 6**).

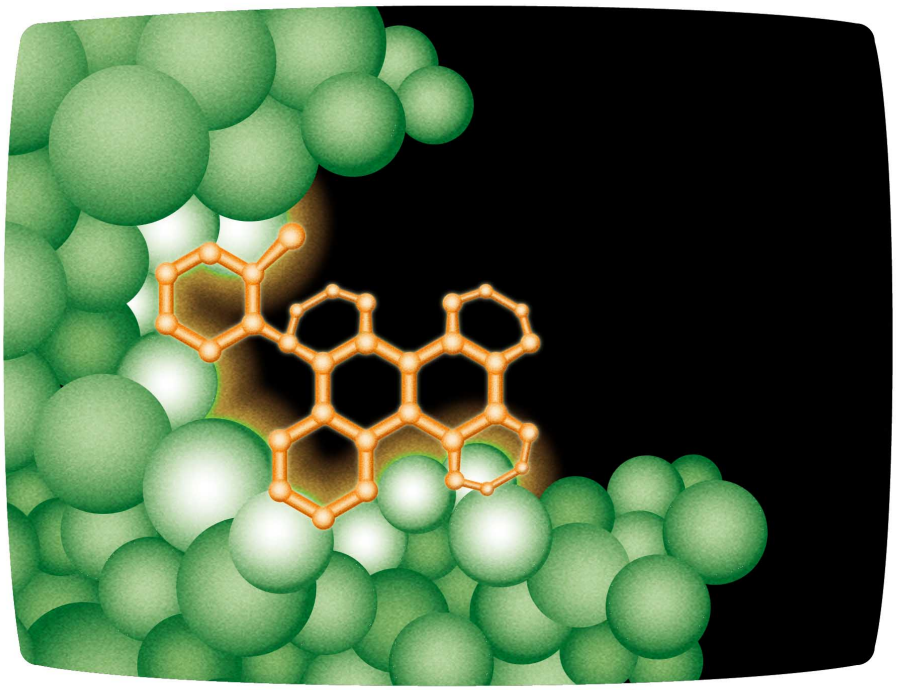


Photo-illustration — Bergmann Graphics

Figure 6. In virtual ligand screening, the three-dimensional image of the protein is fed into a computer, which attempts to fit millions of small molecules to a targeted active site. Small molecules that bind well to the protein become good leads for potential new drugs.

Because many proteins have multiple functions, it may be necessary to develop drugs for each function of a multitask protein. In addition, most proteins act as part of complexes and networks, which may also affect the way a protein acts in a cell. This may also affect the ability of drugs to disable the protein. Understanding the proteome, the structure and function of each protein, and the complexities of protein-protein interactions will be critical for developing the most effective diagnostic techniques and disease treatments in the future.

Ethics and the Economics of Drug Discovery

Drug discovery is simple compared to drug *development*, which requires testing the efficacy and the safety of new drugs through clinical trials. The time (twelve to fifteen years) and cost (approximately 800 million dollars) of drug development are significant economic factors that limit the number of new drugs that come to market; many approved drugs never recover the cost of their development. How do companies decide which promising new drugs to develop? Clearly, there must be very good evidence that the new drug will be effective. But that is not enough; companies also carefully consider the economics of each potential new drug. What is the size of the market for that new drug? How strong is the demand? How effective are current drugs and what are their costs?

The harsh reality of these economics is that new drugs that may benefit only a few are unlikely to make it to clinical trials. Drugs that may benefit millions of people in developing countries too poor to pay for the new drug will also have a low priority for development. While AIDS, malaria, and tuberculosis affect countries that together contain ninety percent of the world's population, only about ten percent of the world's medical research funding is targeted at these diseases. Partnerships among government agencies, charitable organizations, and the pharmaceutical industry may allow companies to allocate some of their resources to developing drugs that will never recover their cost. In 2001 GlaxoSmithKline Biologicals, in partnership with the World Health Organization and the non-profit organization Program for Appropriate Technology in Health, began a program to develop a vaccine for childhood malaria.

Many currently patented drugs could be manufactured in third world countries as generic versions. However, pharmaceutical companies have strongly opposed this practice, fearing that these generic drugs will be inferior to the name brands and would enter the U.S. and European markets at low prices. Brazil has registered generic versions of several AIDS drugs, and manufactures them for itself and other developing countries. In response to worldwide pressure, drug companies have agreed to sell some AIDS drugs at deep discounts to developing countries. However, even with the discounts, the price is much higher than the generic version, limiting the number of AIDS victims who can be treated in poorer nations.

Further Reading

Books

Branden, C., and J. Tooze. 1998. *Introduction to protein structure.* New York: Garland Press.
A non-technical introduction to protein structure.

Ezzell, Carol. 2002. *Scientific American: Beyond the human genome.*
An e-book that describes the new biology after the human genome.

Articles

DeFrancesco, L. 2002. Probing protein interactions. *The Scientist* 16[8]:28.
Researchers have found them by the thousands, but what do these interactions mean?

Ezzell, C. 2002. Proteins rule. *Scientific American* 286:40–48.
Biotech's latest mantra is "proteomics," as it focuses on how dynamic networks of human proteins control cells and tissues.

Hollon, T. 2002. Software zeroes in on ovarian cancer. *The Scientist* 16[8]:16.
A proteomic fingerprint with unprecedented diagnostic accuracy becomes a new kind of disease biomarker.

Hopkin, K. 2001. The post-genome project. *Scientific American* 285:16.
Whether the human proteome will be successfully mapped in three years depends upon how you define "proteome."

Lewis, R. 2002. Fighting the 10/90 gap. *The Scientist* 16[10]:22.
Initiative targets the most neglected diseases; how scientists can help.

McCook, A. 2002. Lifting the screen. *Scientific American* 286:16–17.
An accurate test is not always the best way to find cancer.

Sinclair, B. 2001. Software solutions to proteomics problems. *The Scientist* 15[20]:26.
Researchers find programs to aid every step of research.

Smutzer, G. 2001. Yeast: An attractive, yet simple model. *The Scientist* 15[18]:24.
Researchers use whole genome strategies to characterize unknown genes in yeast.

Stix, G. 1999. Parsing cells. *Scientific American* 287:36.
Proteomics is an attempt to devise industrial-scale techniques to map the identity and activities of all the proteins in a cell.

Glossary

2D gel electrophoresis. A technique for separating proteins to further identify and characterize them. Proteins are separated in the first dimension based on their isoelectric point, and then in the second dimension by molecular weight.

Active site. The specific part of an enzyme that binds the substrate.

Alternative splicing. A biological process in which introns are removed from RNA in different combinations to produce different mRNA molecules from one gene; sometimes called "RNA alternative splicing."

Catalytic domain. The regions of a protein that interact to form the active or functional site of the protein.

Domain. A discrete part of a protein that folds independently of the rest and has its own function.

Domain shuffling. The creation of new proteins by bringing different domains together.

Exon. The sequence of a gene that encodes a protein. Exons may be separated by introns.

Glycosylation. The modification of a protein by adding sugar molecules to particular amino acids in the protein.

High-throughput technology. Large-scale methods to purify, identify, and characterize DNA, RNA, proteins, and other molecules. These methods are usually automated, allowing rapid analysis of very large numbers of samples.

Interaction domain. A discrete module of a protein that is involved in interactions with other proteins.

Intron. The DNA sequence within a gene that interrupts the protein-coding sequence of a gene. It is transcribed into RNA but it is removed before the RNA is translated into protein.

Isoelectric point. The pH at which the net charge of the protein is zero. Proteins are positively charged at pH values below their pI and negatively charged at pH values above their pI.

Kinase. An enzyme that catalyzes the transfer of a phosphate group from ATP to another molecule, often a protein.

Ligand. A molecule that binds to a protein, usually at a specific binding site.

Mass spectrometry. A technique that separates proteins on their mass to charge ratio, allowing identification and quantitation of complex mixtures of proteins.

Motif. A short region in a protein sequence that is conserved in many proteins.

Nuclear magnetic resonance (NMR). A technique for determining the structure of molecules, which is based on the resonance of the nuclei of certain atoms when the molecule is placed in a strong magnetic field.

Peptide mass mapping. A technique for identifying proteins by mass spectrometry; combined with a computer program that matches the information on each peptide's mass against the mass of theoretical, predicted peptides, based on known proteins in databases.

Polyacrylamide gel electrophoresis (PAGE). A technique used to separate proteins in a gel matrix by their relative movement in an electric field.

Phosphatase. An enzyme that removes a phosphate group from a molecule, such as a protein.

Phosphorylation. The addition of a phosphate group to a molecule, such as a protein.

Primary structure. The sequence of amino acids that makes up the polypeptide chain.

Protein fingerprinting. The identification of the proteins in a sample by analytical techniques, such as gel electrophoresis and mass spectrometry.

Protein sorting. The processes in which proteins synthesized in the cytosol are further modified and directed to the appropriate cellular location for their particular function.

Proteome. The complete collection of proteins encoded by the genome of an organism.

Quaternary structure. The association of two or more polypeptides into a larger protein structure.

Secondary structure. The arrangement of the amino acids of a protein into a regular structure, such as an alpha-helix or a beta sheet.

Tertiary structure. The folding of a polypeptide chain into a three-dimensional structure.

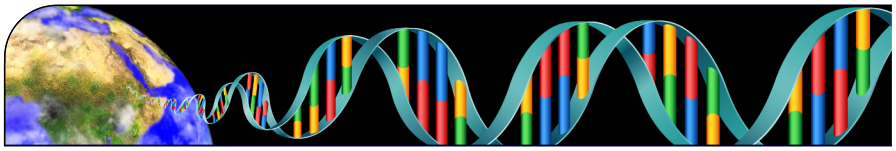
[continues…]

Glossary [continued]

Virtual ligand screening.
A computer-based technology that simulates the interaction between proteins and small molecules to identify those that might be pharmaceutically active and useful as drugs.

X-ray crystallography.
A method for determining the structure of a molecule, such as a protein, based on the diffraction pattern resulting from focused X-ray radiation onto pure crystals of the molecule.

Yeast two-hybrid system.
A method used to identify protein-protein interactions. A protein of interest serves as the "bait" to fish for and bind to unknown proteins, called the "prey."



REDISCOVERING BIOLOGY

Molecular to Global Perspectives

Evolution and Phylogenetics

"Systems of classification are not hat racks, objectively presented to us by nature. They are dynamic theories developed by us to express particular views about the history of organisms. Evolution has provided a set of unique species ordered by differing degrees of genealogical relationship. Taxonomy, the search for this natural order, is the fundamental science of history." STEPHEN J GOULD[1]

Perhaps the most striking feature of life is its enormous diversity. There are more than one million described species of animals and plants, with many millions still left undescribed. (See the *Biodiversity* unit.) Aside from its sheer numerical diversity, organisms differ widely and along numerous dimensions — including morphological appearance, feeding habits, mating behaviors, and physiologies. In recent decades, scientists have also added molecular genetic differences to this list. Some groups of organisms are clearly more similar to some groups than to others. For instance, mallard ducks are more similar to black ducks than either is to herons. At the same time, some groups are very similar along one dimension, yet strikingly different in other respects. Based solely on flying ability, one would group bats and birds together; however, in most other respects, bats and birds are very dissimilar. How do biologists organize and classify biodiversity?

In recent decades, methodological and technological advances have radically altered how biologists classify organisms and how they view the diversity of life. In addition, biologists are better able now to use classification schemes for diverse purposes, from examining how traits evolve to solving crimes. These advances have strengthened evolutionary biology as a theory: a theory in the scientific sense, meaning a "mature coherent body of interconnected statements, based on reasoning and evidence, that explains a variety of observations."[2] Molecular biology, genetics, development, behavior, epidemiology, ecology, conservation biology, and forensics are just a few of the many fields conceptually united by evolutionary theory.

A Brief History of Classification

Taxonomy, the practice of classifying biodiversity, has a venerable history. Although early natural historians did not recognize that the similarities and differences among organisms were consequences of evolutionary mechanisms, they still sought a means to organize biological diversity. In 1758 Carl Linné proposed a system that has dominated classification for centuries. Linné gave each species two

names, denoting genus and species (such as *Homo sapiens*). He then grouped genera into families, families into orders, orders into classes, classes into phyla, and phyla into kingdoms. Linné identified two kingdoms: Animalia (animals) and Plantae (plants). Biologists generally accepted the idea of evolution shortly after the publication of Darwin's *The Origin of Species* and, since Linné's classification system, they have described an immense number of species. Despite these facts, taxonomy changed little until the 1960s.

The first major break from the Linnean model came from Thomas Whittaker. In 1969 Whittaker proposed a "five kingdom" system in which three kingdoms were added to the animals and plants: Monera (bacteria), Protista, and Fungi. Whittaker defined the kingdoms by a number of special characteristics. First, he specified whether the organisms possessed a true nucleus (eukaryotic) or not (prokaryotic). Because Monera are prokaryotic and virtually all are unicellar, they are distinct from the other four eukaryotic kingdoms. With few exceptions, the eukaryotic unicellular organisms were placed into the kingdom Protista.

The three multicellular eukaryotic kingdoms distinguish themselves by the general manner in which they acquire food. Plants are autotrophs and use photosynthetic systems to capture energy from sunlight. Animals are heterotrophs and acquire nutrients by ingesting plants or other animals, and then digesting those materials. Fungi are also heterotrophs but, unlike animals, they generally break down large organic molecules in their environment by secreting enzymes. Unicellular organisms use a variety of modes of nutrition. (See the *Microbal Diversity* unit.)

The five kingdoms system was certainly an advance over the previous system because it better captured the diversity of life. Three groups — bacteria, fungi, and protists — did not fit well into either the animal or plant category. Moreover, each of these three groups appeared to possess diversity comparable to that of animals or plants. Thus, the designation of each as a kingdom seemed fitting.

In the years since Whittaker's system was developed, however, new evidence and new methods have shown that the five-kingdom system also fails to adequately capture what we now know about the diversity of life. Microbial biologists became aware of these limitations as they discovered unicellular organisms that appeared to be prokaryotic, but were extremely distinct in ultrastructure and other characteristics from the traditional bacteria. Some of these unusual prokaryotes lived in hot springs and other places where the temperatures were near, or even above, the boiling point of water (the thermophiles). Others, the extreme halophiles, were able to tolerate salt concentrations as high as five Molar, roughly ten times the concentration of seawater. (See the *Microbal Diversity* unit.) DNA sequence data also increasingly suggested that these prokaryotes were most unlike the traditional bacteria.

The microbal evolutionist Carl Woese proposed a radical reorganization of the five kingdoms into three domains. (See the *Microbial Diversity* unit.) Starting in the 1980s Woese's scheme has been increasingly accepted by evolutionary biologists and is now the standard paradigm. In his classification system, Woese placed all four eukaryotic kingdoms into a single domain called **Eukarya,** also known as the eukaryotes. He then split the former kingdom of Monera into

the Eubacteria (bacteria) and the Archaea (archaebacteria) domains. Woese then placed most of the "unusual" prokarytes in the Archaea, leaving traditional bacteria in the Eubacteria. The Woese classification represents a demotion of the animals and plants as individual kingdoms. This is consistent with recent discoveries of more diversity among microbes than between animals and plants.

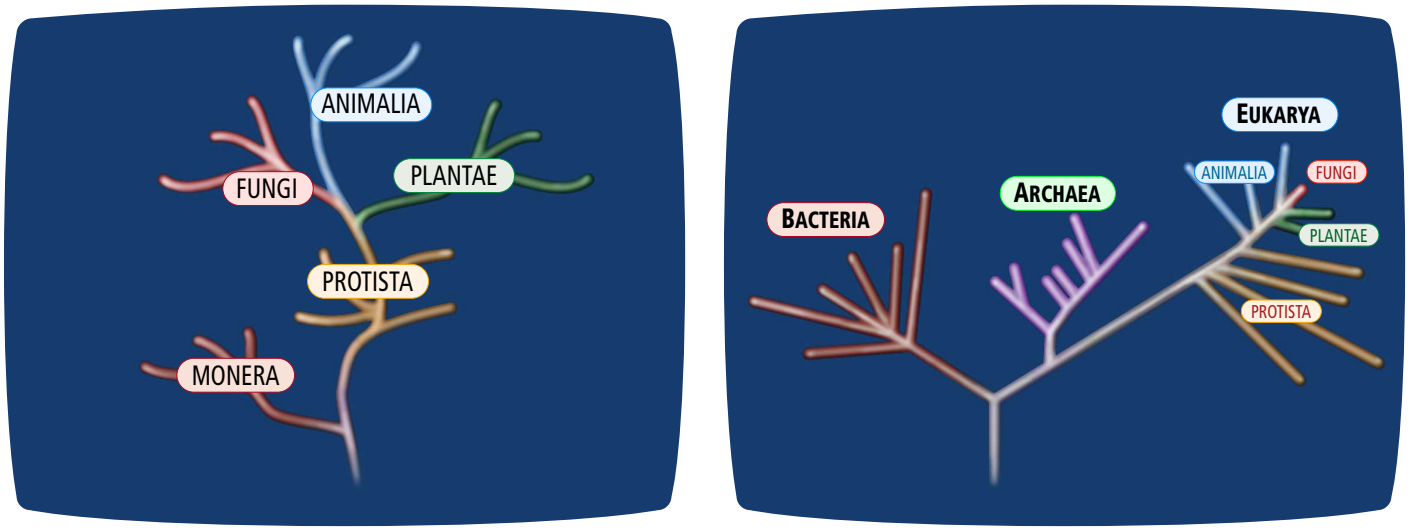


Photo-illustration — Bergmann Graphics

Figure 1. The older five-kingdom tree of life, which has been replaced by Woese's three-domain tree.

Unlike Whittaker's five kingdoms system, Woese's three domains system organizes biodiversity by evolutionary relationships. After a discussion of the methodology of contemporary evolutionary classification, we will examine the methods Woese used and the justification for his system.

Cladistics and Classification

Except for his last sentence where he used the word "evolved," Charles Darwin never mentioned "evolution" in *The Origin of Species.* Instead, he used the phrase "descent with modification." Evolutionary classification today is based on those two central features of evolution: groups of organisms descend from a common ancestor and, with the passage of time, acquire modifications.

Cladistic analysis, also known as cladistics and phylogenetic systematics, is the main approach of classification used in contemporary evolutionary biology. The German taxonomist Willi Hennig developed cladistics in 1950, but his work was not widely known until it was translated into English in 1966. After scientists began using molecular data in classification, Hennig's cladistics became increasingly adopted.

Cladistic analysis starts with the assumption that evolution is a branching process: ancestral species split into descendant species, and these relationships can be represented much like family trees represent genealogies. The "trees" obtained by such analyses are called **phylogenies.** These phylogenies should be viewed as testable hypotheses, subject to either confirmation or rejection depending on new evidence. Of course, hypotheses differ as to how much support they have. Some are so well supported (such as that humans share a closer common ancestor to chimpanzees than either share with lemurs) that they are exceedingly unlikely to be overturned.

In cladistic analysis, groups of organisms, known as **taxa**, are arranged into **clades** that are then nested into larger clades. The term "taxa" (singular "taxon") can be applied to groups of any size. Taxa that are each others' closest relatives are called **sister taxa**. Each clade should be **monophyletic**; that is, all members share a single common ancestor, and all descendants of that ancestor are included in the clade. In contrast, a **polyphyletic** group is one in which the members are derived from more than one common ancestor. What if all of a particular clade's members share a common ancestor but not all taxa that share that common ancestor are included in that group? Such a group is called **paraphyletic**.

Taxonomists following cladistic analysis place taxa into clades based on the derived character states that the taxa share. For example, a wing is a character. The presence or absence of a wing would be alternative character states. Other features of a wing (such as its shape and size, and how it develops) could also be character states. Aside from the presumption that characters are independent of one another, any trait can be a character. In principle, there is no difference between the analysis of morphological and molecular characters. The characters used most often in molecular phylogenies are the nucleotide positions of the examined DNA molecule(s); thus, the character states are the actual nucleotides at that position. Shared, derived characteristics are known as **synapomorphies**.

That taxonomists would classify taxa based on similarity makes sense. After all, like goes with like. But why would they consider only the derived shared character states? Why not consider all character states, including those that are primitive? The rationale is that the primitive characters do not reveal information about which groups share more recent common ancestors; the primitive character states would only contribute noise to the system. In classifying different groups of birds that all fly, whether they fly does not contribute information. In fact, in classifying flightless birds, considering the ancestral state (flighted) can actually distort the obtained phylogeny away from the true phylogeny. For these reasons, only synapomorphies (shared, derived character states) are considered in the analysis. In practice, taxonomists often have difficulty in distinguishing between which character states are primitive and which are derived.

For what reasons can taxa share synapomorphies? One possibility is that they share a common ancestor. This is called **homology**. While cladistic analysis assumes that most synapomorphies will arise by homology, they can arise by other ways. One possibility is **convergence:** different lineages that do not share a recent common ancestor evolve to the same character state. An obvious example is that both bats and birds have wings; however, these were independently derived, most likely owing to similar selective forces. This example is obvious because so many other characters place bats closer to non-winged clades (other mammals) than to birds. Yet, less obvious cases can be resolved only after cladistic analysis. Another possible reason why non-homologous character states can be similar is a **reversal** in which mutation or selection causes the derived character state to revert to the ancestral state.

How does cladistic analysis work, especially given the possibility of conflicting data generated by reversals and convergence? Taxonomists, like scientists in general, start with the principle of parsimony — that

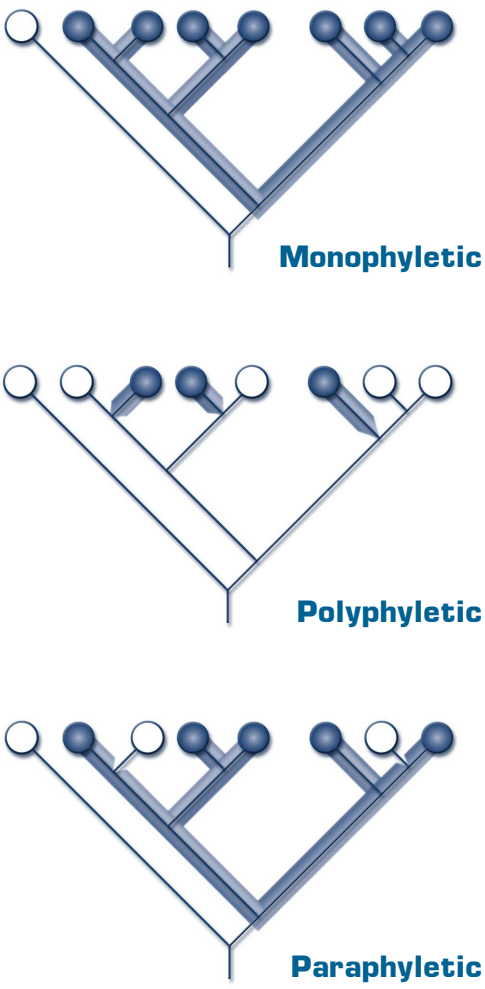


Figure 2. Examples of monophyletic (top), polyphyletic (middle), and paraphyletic (bottom) trees.

the shortest, most simple, and direct path is most likely to be the correct one. In one commonly used method, **parsimony analysis**, the taxonomist searches for the most parsimonious tree; that is, the one that requires the fewest number of evolutionary transitions. Consider the example in Figure 3: three possible phylogenies exist. Based on the data given, for the top phylogeny to occur, we must postulate a total of nine evolutionary changes. The middle phylogeny requires postulating ten changes, and the lower phylogeny requires postulating eleven changes. Because the first phylogeny requires the fewest changes, it is the most parsimonious tree.

The most parsimonious tree may not necessarily represent the true phylogenetic relationships. Perhaps certain types of transitions are more likely or evolved more easily than are others. It is often difficult to know before doing the analysis, which changes are most likely. Thus, taxonomists generally resort to the fallback position that all changes are equally likely. There are some cases, particularly with molecular data, where there is good prior knowledge of variation in the likelihoods of different changes. For instance, certain types of mutations are more likely than others are. Transitions (changes from a purine — A or G — to the other purine, or a pyrimidine — C or T — to the other pyrimidine) are more likely than transversions (changes from a purine to a pyrimidine or vice-versa). Using increasingly statistical techniques, such as maximum likelihood analysis, taxonomists can adjust for these situations.

Figure 4 shows an example of an **unrooted tree**. Unrooted trees do not display the directionality of evolution, only patterns of relatedness. A unrooted tree can be rooted, but for any given unrooted tree there are many possible **rooted trees** that can be derived. Rooting a tree usually requires identification and use of an **outgroup** — a taxon that is more distantly related than the taxa contained within the tree. For instance, given an unrooted tree containing the great apes (humans, chimpanzees, gorillas, orangutans, and gibbons), one could use a species of monkeys, such as baboons, as an outgroup. (See the *Human Evolution* unit.) In practice, taxonomists often use multiple outgroups to refine the analyses.

	TAXA			
Characteristics	1	2	3	4
A	+	+	+	–
B	+	–	–	–
C	+	–	+	–
D	+	+	–	–
E	+	+	+	–
F	+	–	+	+
G	+	+	–	–
H	+	+	+	–

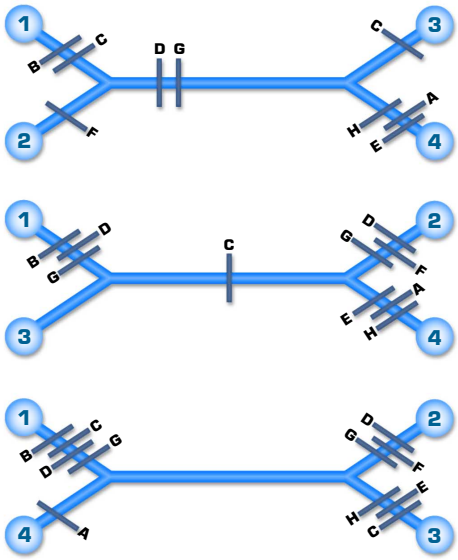


Figure 3. Three possible unrooted trees are shown. The top tree assumes nine changes in character state occurred (each change is represented by a mark), the middle tree assumes ten changes, and the bottom tree assumes eleven. Because the top tree assumes the fewest changes, it is the most parsimonious tree.

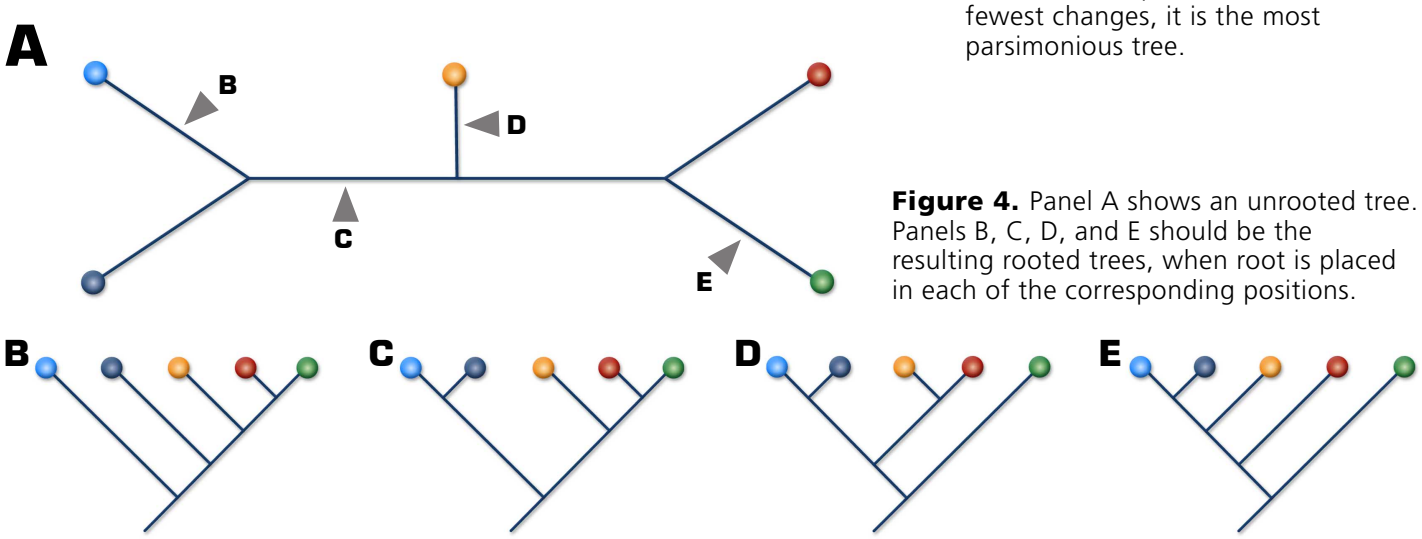


Figure 4. Panel A shows an unrooted tree. Panels B, C, D, and E should be the resulting rooted trees, when root is placed in each of the corresponding positions.

Applications of Molecular Phylogenetics

Although the methods used in cladistic analysis are the same for both molecular and morphological characters, molecular data provides several advantages. First, molecular data offers a large and essentially limitless set of characters. Each nucleotide position, in theory, can be considered a character and assumed independent. The DNA of any given organism has millions to billions of nucleotide positions. In addition, the large size of the genome makes it unlikely that natural selection will be strongly driving changes at any particular nucleotide. Instead, most nucleotide changes are "unseen" by natural selection, subject only to mutation and random genetic drift. If we were to assume that the driving force of natural selection is less prevalant for molecular characters, then we should assume that the probability of convergence for molecular characters is also.

By selecting a particular class of morphological characters, researchers may also bias the analysis in such a way that groups with certain characteristics cluster with others for reasons other than homology. For instance, if the set of characters were weighted toward those involved in carnivory, carnivorous animals may cluster together — not because of homology but because of shared function. This problem would be less likely if using molecular characters.

Another advantage of molecular data is that all known life is based on nucleic acids; thus, studies involving any type of taxa can use DNA sequence data. Some genes or regions of genes evolve quickly. These are most useful in studies of closely related taxa. Conversely, other genes (or regions) are slower to evolve. These are the most useful for studies of more distantly related organisms. At the extreme, some evolutionarily related genes have been found in disparate organisms such as yeast and humans. Rates by which sections of DNA evolve are primarily determined by the extent of functional constraint. Genes and positions within genes that are the most useful generally evolve the slowest. This is because they are the least able to tolerate mutational change without substantially reducing the fitness of the individuals that harbor them. Many of these very conserved genes play a role in development. (See the *Genes and Development* unit.)

Starting in the late 1970s Carl Woese took on an ambitious project — determining the relationships of all life, which resulted in the reorganization of the tree of life. To do this, Woese and his associates took advantage of a molecule that evolves extremely slowly — (rDNA) the DNA that encodes a small subunit of ribosomal RNA. They found that the sequences cluster in three groups corresponding to the eukaryotes (Eukarya), the archaea, and the eubacteria. We discussed these three domains earlier.

The three-domains model was controversial for several reasons. First, the conclusions Woese drew were initially based on evidence from a single gene. Perhaps there was something unusual about the way that small subunit of rDNA evolved, his critics said. That controversy was easily solved by generating more data. Sequences from other genes that evolve slowly seemed to confirm the rationale for the three domains. A more fundamental problem was that Woese's tree was unrooted. If each domain represents a monophyletic group, three possiblilties existed: (1) that the eubacteria and archaea are sister groups, with the eukaryotes branching off first; (2) that eubacteria and

eukaryotes are sister groups; or (3), that archaea and eukaryotes are sister groups. Woese himself suspected this third possibility. A fourth possibility was that the root of the tree lied within one of the domains and, therefore, the domain was not monophyletic. To root a tree, one generally requires an outgroup. But what is the outgroup to all known life? Rocks?

Margaret Dayhoff proposed an ingenious solution to this rooting dilemma: using ancestral genes that are present in multiple copies in the same organism because of gene duplication. If there were such genes that had duplicated before the split among the three domains, these could be used as outgroups to root the tree of life. In 1989, many years after Dayhoff's suggestion, Naoyuki Iwabe and colleagues used this approach.[3] Organisms in all three domains have two distinct genes that code for the two subunits (alpha and beta) of the enzyme that hydrolyzes ATP to yield energy, ATPase. DNA sequence similarity strongly suggests that these two genes are derived from a gene duplication predating the divergence of the domains. The ATPase-alpha tree, using an ATPase-beta gene as an outgroup, showed that each of the domains was monophyletic, and that eukaryotes and archaea are sister groups. The same result was obtained when ATPase-beta was used as an outgroup to root the ATPase-alpha tree. Similar trees were obtained with other pairs of duplicated genes. In conclusion, Woese was right.

HIV and Forensic Uses of Phylogenetics

Phylogenetic methods have been used to solve practical problems, including determining the sources of infection from HIV. This retrovirus evolves at an extremely rapid rate, owing to its exceptionally high mutation rate. In fact, sequences of HIV genes taken from the same infected individual can be as different as sequences from some homologous genes in humans and birds. Its rapidity of evolution works to HIV's advantage as it wreaks havoc on the immune system. On the other hand, scientists can take advantage of that rapid evolution to study the relationships between HIV and other similar viruses.

Researchers at the Centers for Disease Control and Prevention (CDC) used phylogenetic systematics of HIV for forensic purposes. During the early 1990s a Florida dentist was suspected of transmitting HIV to several of his patients. After the first case of probable transmission surfaced, the dentist wrote an open letter to his patients suggesting that they be tested for HIV. At least ten of the patients tested positive for HIV. However, a few of the infected individuals had other risk factors; therefore, there was the distinct possibility that they had not been infected by the dentist. The CDC researchers sequenced the HIV gp120 gene from several viral isolates taken from the dentist, his infected patients, and non-patients who were also infected. From the phylogeny constructed based on the HIV sequence data, they first denoted what they called the "dentist clade." This monophyletic group contained sequences from the HIV sequences collected from the dentist but not from the non-patients. Five of the patients had viral sequences that were contained in the dentist clade. These patients also lacked other risk factors. Thus, by strong inference, the CDC researchers determined that the dentist had infected these five patients.

There was some controversy over whether or not the dentist clade identified in the CDC study was reliable. Nucleotides in the HIV gp120 gene do not evolve in same way as in other genes. Instead of transitions being universally more prevalent than transversions, as is the case in most genes, A to C transversions are more frequent than transitions of C to T. There was also concern about the types of algorithms used. To address these concerns, David Hillis, John Huelsenbeck, and Cliff Cunningham re-analyzed the data of the CDC study. They found that, under nearly all circumstances, the same dental clade was obtained.[4] Thus, the results were statistically reliable. Investigators are using similar studies to determine the source of the anthrax used in the attacks of October 2001.

The Origin of Bats and Flight

Molecular phylogenetics are often most useful when there is conflict among the phylogenies constructed with different morphological character data sets. For instance, molecular data have helped settle the question of whether bats are a monophyletic group — that is, whether they share a common ancestor not shared by non-bats. In the 1980s several morphological analyses challenged the traditional view that bats (order Chiroptera) were monophyletic. The studies proposed that the large fruit-eating Megachiroptera (megabats) were actually more closely related to primates than they were to the smaller insect-eating Microchiroptera (microbats). The studies based the megabat-primate grouping on synapomorphies that included features of the penis, brain, and limbs. The implication of this reclassification was that flight evolved more than once within mammals.

Spurred by this controversy, several research groups performed cladistic analyses of bats using molecular data during the early 1990s. For example, Loren Ammerman and David Hillis sequenced mitochondrial DNA sequences from many mammals, including two species of microbats, two species of megabats, a tree shrew, a primate, and several outgroups. From their data, the most parsimonious tree that assumed bat monophyly was ten steps shorter than the most parsimonious tree that assumed bats were not monophyletic. Statistical analysis showed that bat monophyly was significantly more parsimonious than the absence of bat monophyly.

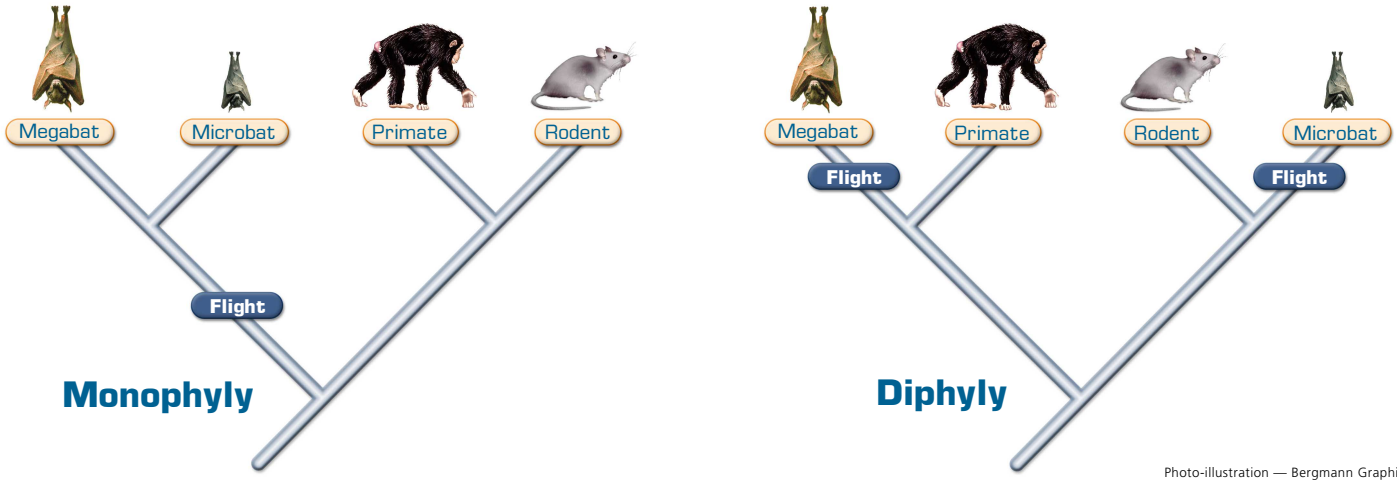


Photo-illustration — Bergmann Graphics

Figure 5. Alternative possibilities of bat phylogeny. **Left:** Bats form a monophyletic clade, in which flight evolved once in mammals. Alternately, **right**, bats are diphyletic, and flight evolved twice in mammals.

Other molecular phylogenetic studies, using a variety of different classes of genes, showed the same pattern of bat monophyly. These researchers also indicated that convergence is the most likely reason why some derived morphological character states seem to be shared by primates and bats.[5]

Other researchers raised the objection that these early molecular phylogenetic studies did not take into account biases in the way that sequences evolve. Specifically, the critics noted that both microbats and macrobats have DNA with a higher proportion of G's and C's than A's and T's. It is well known that organisms that have higher metabolic rates will have higher G-C content. Thus, the critics argued, perhaps the apparent monophyly of bats that was observed in the molecular studies is due to convergent evolution toward high G-C content and not homology. Using various methods, subsequent molecular phylogenetic studies took the bias in nucleotide changes into account. One simple method was to split the DNA sequences into A-T rich and G-C rich regions and do a separate analysis on each. Even after nucleotide sequence bias was discounted, the most parsimonious phylogenies still showed that all bats had a single common ancestor. This support for bats as a monophyletic group is also strong evidence for flight evolving only once in mammals.

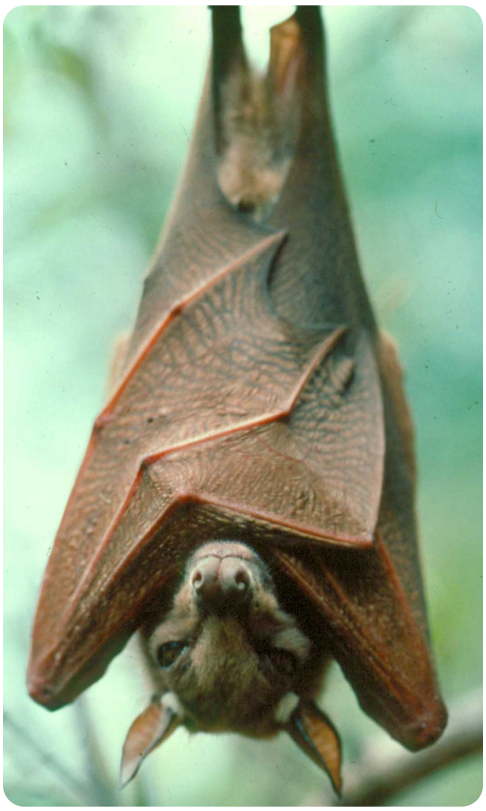
The monophyly of bats is an example where molecular data shored up the traditional phylogeny against challenges posed by some morphological characters. In contrast, there are also occasions where analysis of the molecular data provided an unexpected answer. One such example is the example of the evolutionary history of whales, which is discussed in detail in the video.

Challenges

There have been tremendous advances in comparative evolution brought on by the new methods of phylogenetic analysis and burgeoning amounts of DNA sequence data; however, the field is not without challenges and limitations. Some of these challenges are due to features of the organism and some are due to limitations of the tools we currently possess.

One feature of the organism that presents a challenge is the horizontal transfer of genes across different species. In the standard mode of vertical transmission, genes are transmitted from parent to offspring (whether by sexual or asexual means). Genetic material can also be exchanged among different organisms, especially bacteria. This general type of transmission is called **lateral (horizontal) gene transfer**. One mode by which lateral gene transfer can occur is **conjugation**, whereby some bacteria exchange genes (plasmids or small parts of the bacterial chromosome) by physical contact. Bacteriophages can also mediate lateral gene transfer by cross-infection. Amazingly, these processes that result in lateral gene transfer can occur among bacteria that differ by as much as fifteen percent at the DNA sequence level. The implication of widespread and random lateral transfer of genes is that the genetic structure of bacteria can be mosaic — different genes or gene regions may have different histories. If lateral transfer is sufficiently pervasive, it could lead to the inability of constructing the true phylogeny for all bacteria. (See the *Microbial Diversity* unit.)

Figure 5a. Photographs of an example of a megabat, the African fruit bat, and a microbat, the Mexican freetail bat.



Courtesy of the Transvaal Museum,South Africa.

Courtesy of Robert Bloomberg.

The most dramatic case of lateral gene transfer involving eukaryotes is the endosymbiotic origin of mitochondria. This view, championed by Lynn Margulis, speculates that these ATP-producing organelles were once free-living prokaryotes that were engulfed by a proto-eukaryote — an idea now strongly supported. The evidence includes similarities of ribosomal structure, sensitivity to antibiotics, and DNA sequences between mitochondria and prokaryotes. The major controversy is how and when this process occurred. Other eukaryotic organelles have been shown to probably have endosymbiotic origins. The conventional wisdom, however, is that lateral gene transfer involving eukaryotes was limited from these exceeding rare endosymbioic events.

Recent evidence strongly suggests that lateral gene transfer involving eukaryotes may be more prevalent than once thought. In some DNA sequences, bacterial or archaeal sequences cluster in clades that are otherwise strictly eukaryotic. The extent to which lateral gene transfer among the kingdoms and within the eukaryotes has occurred is still a matter of controversy and inquiry. The implications for our ability to construct accurate phylogenies for these "deep" relationships are also controversial. There appears to be a continuum of the degree to which different genes transfer across distantly related taxa. Some researchers have argued that we may be able to get around the problem of lateral gene transfer by choosing genes that display very little — if any — horizontal gene transfer.

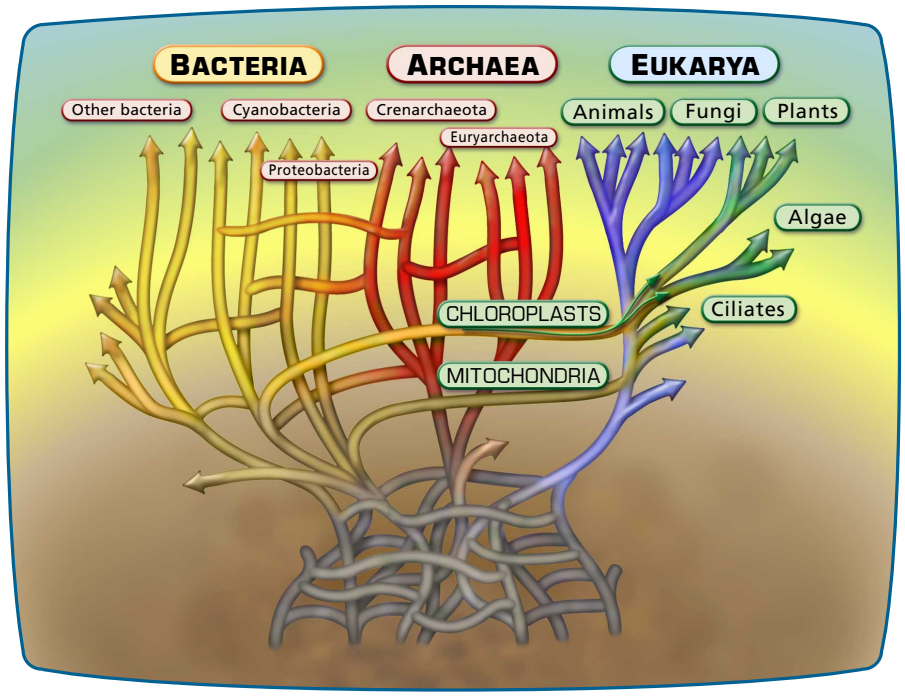


Photo-illustration — Bergmann Graphics

Figure 6. This view of early evolution suggests multiple primitive cells as ancestors to the three domains, and illustrates lateral gene transfer among early organisms.

Another major challenge to comparative evolution is that the methodology of phylogenetic systematics is computationally extensive. The number of potential trees increases extremely quickly — faster than exponentially — as the number of taxa increases. For three taxa, there are only three possible rooted trees. For a given data set, one can readily determine by inspection which tree is the most parsimonious. Given seven taxa, it would be exceedingly painstaking for a person to search for the most parsimonious tree through the

10,395 rooted possibilities; however, a desktop computer with the correct software could search among all of these possibilities in a tiny fraction of a second.

Increasing computing power alone will not solve this problem. At twenty taxa, the number of possible rooted trees exceeds 8 times 10^{21} — a number of similar magnitude to the total number of cells in all living human beings. Soon after this point, it becomes impractical for computers to search through all the possibilities to find the most parsimonious one. Given fifty taxa, it would take literally longer than the age of the universe to search through every single possible unrooted tree — even if computers were a million times faster than they are now. Therefore, phylogenetic systematics must employ methods other than searching every single possible tree when evaluating data sets that involve a large number of taxa. One method is to collapse taxa that are known (by other information) to be close relatives into a single taxon to make the analysis more feasible. Researchers have also used various searching approaches, sometimes called heuristics. This approach uses algorithms to identify regions of "tree space" that are likely to contain very parsimonious trees. These heuristic methods may not always identify the best tree, but they will identify trees that are nearly as parsimonious as the best tree most of the time.

Coda: The Renaissance of Comparative Biology

We are witnessing a renewal of interest in comparative approaches to studying function. Biology in the 1800s was almost entirely comparative. In the twentieth century we moved into a strongly reductionistic period of genetics, developmental biology, and physiology. This trend only intensified with the rise of molecular biology, particularly after the elucidation of the structure of DNA in 1953. At that time, comparative biology was marginalized as just "natural history." At the turn of the twenty-first century comparative approaches have staged a strong comeback. In large part, this renaissance is due to the revolution in data gathering (particularly of DNA sequences) and the effort already devoted to establishing particular model systems. In contrast to the comparative biology of ninteenth century, today's comparative evolutionary biology rests on a strong foundation of functional genetics.

References

1) Gould, S. J. 1987. *Natural history*.

2) Futuyma, D. J. 1998. *Evolutionary biology*. 3d ed. Sunderland MA: Sinauer Press, p. 11.

3) Iwabe, N., K. Kuma, M. Hasegawa, S. Osawa, and T. Miyata. 1989. Evolutionary relationship of the archebacteria, eubacteria, and eukaryotes inferred from phylogenetic trees of duplicated genes. *Proceedings of the National Academy of Sciences* 86:9355–59.

4) Hillis, D. M, J. P. Huelsenbeck, and C. W. Cunningham. 1994. Application and accuracy of molecular phylogenies. *Science* 264:671–77.

5) Ammerman, L. K., and D. M. Hillis. 1992. A molecular test of bat relationships: Monophyly or diphyly? *Systematic Biology* 41:222–32.

Further Reading

Books

Freeman, S., and J. C. Heron. 2001. *Evolutionary analysis*. 2d ed. Upper Sable River, NJ: Prentice Hall.

An excellent inquiry-based college-level textbook on evolution. It is somewhat more accessible than Futuyma's textbook.

Futuyma, D. J. 1998. *Evolutionary biology*. 3d ed. Sunderland, MA: Sinauer Press.

This is perhaps the most comprehensive textbook on evolutionary biology. It also provides an excellent entry into the primary literature of evolutionary biology.

Article

Hillis, D. M, J. P. Huelsenbeck, and C. W. Cunningham. 1994. Application and accuracy of molecular phylogenies. *Science* 264:671–77.

A technical review of the state of phylogenetic systematics as of the middle 1990s.

Glossary

Clade. An organizational term used in cladistics to describe a group of related organisms being compared.

Conjugation. Cell-to-cell contact in which DNA copied from a plasmid or chromosome is transferred to a recipient cell. It can contribute to lateral gene transfer when it occurs between distantly related bacteria.

Convergence. The phenomenon where more distantly related lineages have similar features due to the operation of similar evolutionary forces.

Eukarya. The domain of all eukaryotic organisms. Eukaryotes are single or multicellular organisms with cells that have a membrane-enclosed nucleus and usually other organelles.

Homology (homologous). Similarity of genes or other features of organisms due to shared ancestry.

Lateral gene transfer (Also referred to as *horizontal gene transfer.)* The transmission of genes directly between organisms, particularly bacteria, and not from parent to offspring.

Monophyletic. A clade, or group, of organisms that includes every member of the group and its shared common ancestor.

Outgroup. An unrelated group or organism used for the purpose of comparison.

Paraphyletic. An incomplete clade of related organisms from a common ancestor.

Parsimony analysis. A method used to create phylogenies of organisms based on the assumption that the evolution of characters occurs by the simplest (most parsimonious) path.

Phylogeny. A tree-like diagram used to represent evolutionary relationships between species or groups.

Polyphyletic. A clade containing related groups of organisms derived from several unrelated ancestors.

Reversal. A phenomenon wherein mutation or selection causes a derived character state to revert back to the ancestral state.

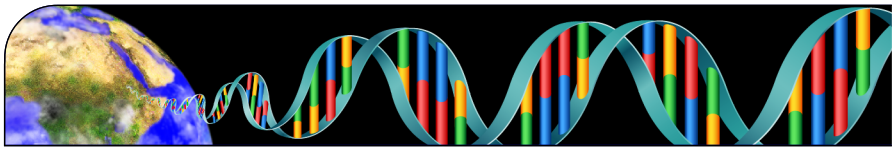
Rooted tree. A phylogeny in which the evolutionary ancestor is known.

Sister taxa. The most closely related groups of organisms in a phylogeny.

Synapomorphies. Derived character states that are shared by two or more taxa.

Taxa. Groups or representatives of related organisms that are being compared; they can vary in hierarchical level (such as genus, family, order, and so on).

Unrooted tree. A phylogeny in which the evolutionary ancestor is not known.



REDISCOVERING BIOLOGY

Molecular to Global Perspectives

Microbial Diversity

"If we look we'll find 'em… the microbes are there. They're these little packages of secrets that are waiting to be opened." ANNA-LOUISE REYSENBACH, PHD

Introduction

Microbes flourish. Inside your gut, in the mucky soil of a marsh, in Antarctic ice, in the hot springs of Yellowstone, in habitats seemingly incompatible with life, microbes flourish.

They were present on Earth 3.5 to 4 billion years ago, and they've been evolving and expanding into new environments ever since. Replicating quickly, exchanging genetic material with each other and with other organisms, bacteria and **archaea** have become ubiquitous.

Not only are they everywhere, but these tiny organisms also manipulate the environments in which they live. Their presence has driven the development of new ecosystems — some of which allowed for the evolution of more complex organisms. Without microbes, the recycling of essential nutrients on Earth would halt. Microbes communicate; some generate the signals for the formation of metabolically diverse communities. Some use sophisticated signaling to establish complex relationships with higher organisms.

In this unit we will examine examples of the broad diversity of microorganisms and consider their roles in various ecosystems, both natural and man-made. We will also discuss some of the practical applications that derive from the wealth of metabolic diversity that microorganisms possess.

Let's start at the beginning… three or four billion years ago.

Microbes as the First Organisms

No one knows for certain where life began. Hot springs and volcanic (hydrothermal) vents on the ocean floor, however, may represent the kinds of environments where cellular life began. Before the ozone layer formed, the surface of Earth was exposed to strong radiation. Thus, most of the Earth's earliest organisms probably developed beneath the terrestrial surface or in the oceans. It's likely that these early microbes adapted to the high temperatures associated with abundant volcanic activity. Geological turmoil resulted in the accumulation of carbon dioxide in the atmosphere.

Sometime later, about 2 or 2.5 billion years ago, gaseous oxygen began to appear. Unlike the carbon dioxide, oxygen almost certainly came

about because of microbes. Microbes similar to today's cyanobacteria were present at this time. We know this based on the presence of **stromatolites** — fossilized microbial mats consisting of layers of filamentous prokaryotes — and trapped sediment that date back to that time. Stromatolite-forming bacteria obtain carbon from carbon dioxide and get their energy by photosynthesis, splitting water to generate oxygen gas in the process. These organisms brought the oxygen level in Earth's atmosphere to about ten percent of what it is today — enough to allow the evolution of oxygen-using organisms. Gaseous oxygen also contributed to the formation of the ozone layer, which blocks UV radiation. New terrestrial habitats were now open for an evolving diversity of microbes.

The Diversity of Microbial Metabolism

The diverse environments on Earth today present energy, and carbon and other nutrients in varying forms. They also vary with respect to temperature, acidity, and the availability of byproducts from other organisms. Microbes thrive in a vast array of these environments.

Microorganisms vary with regard to the sources of energy they use for assembling macromolecules and other cellular components from smaller molecules. Phototrophs obtain their energy from light; chemotrophs use chemicals as energy sources. (*Troph* is derived from a Greek word meaning "to feed.") Many organisms use organic compounds as sources of energy; these are the **chemoorganotrophs**. In contrast, the chemolithotrophs use inorganic chemicals as energy sources.

Microorganisms also vary with respect to the source of carbon they use. Autotrophs are able to build organic molecules from carbon dioxide. Heterotrophs, the "other feeders," obtain their carbon from organic compounds — amino acids, fatty acids, sugars, and so on — of autotrophs.

These terms are often combined. So, a "photoautotroph" is an organism that, like plants, gets its energy from light and its carbon from CO_2. Decomposers are often chemoheterotrophs; they may obtain energy and carbon from the same source.

Bacterial Energy Sources	
Phototroph	Light
Chemotroph	Chemicals
Chemoorganotroph	Organic compounds
Chemolithotroph	Inorganic compounds

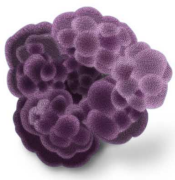
Bacterial Carbon Sources	
Autotroph	Carbon dioxide
Heterotroph	Organic compounds

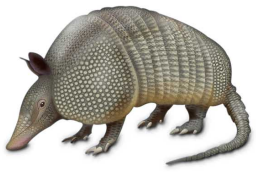
So what metabolic classes might microbes found in a deep-sea hydrothermic vent fall within? The lack of sunlight makes them dependent on chemical energy; thus, they are chemotrophs. Carbon dioxide dissolved in the ocean is their source of carbon; they are autotrophs. Organic material from decomposing phototrophs is not abundant, so these organisms rely on inorganic sources for energy. They may use H_2 (present in magmatic gases), reduced sulfur compounds, or methane as a source of energy. They are also **thermophiles**, growing optimally at temperatures above 45°C. Thermophilic **chemolithoautotrophs** serve as primary producers, the first organisms in food chains that include animals such as tube worms and giant clams.

Archaea and Bacteria

As reviewed in "Evolution and Phylogenetics," living organisms can be grouped into three domains: the Archaea, the Bacteria, and the **Eukarya**. Members of Bacteria and Archaea are prokaryotes: single-celled organisms lacking true nuclei and other membrane-enclosed organelles. Bacteria and archaea, however, differ in cell wall characteristics and membrane lipid composition. They also differ in RNA polymerase structure and, therefore, protein synthesis.

Many **extremophiles** (organisms that tolerate high or low temperature, high salinity, or extreme pH) fall within the Archaea. Some archaea, the extreme halophiles (salt lovers), tolerate salt concentrations as high as nearly ten times that of seawater. They have also been found thriving in the Great Salt Lake and the Dead Sea. Nevertheless, habitat alone does not differentiate the groups. Some bacteria grow at temperatures above 80°C, and some archaea have been found in environments not considered extreme. For example, **methanogenic** archaea live in anoxic sediments in marshes and are used in sewage treatment facilities. Another archaean, *Methanobrevibacter smithii,* lives and generates methane in the human colon.





	BACTERIA	ARCHAEA	EUKARYA
Cell type	Prokaryotic	Prokaryotic	Eukaryotic
Cell wall	Contains peptidoglycan	Lacks peptidoglycan	If present contains no peptidoglycan
Plasma membrane lipids	Ester links between polar heads and fatty acid tails	Ether links	Ester links
RNA polymerase	One (4 subunits)	Several (8-12 subunits each)	Three (12-14 subunits each)
Initiator tRNA	Formylmethionine	Methionine	Methionine

Photo-illustration — Bergmann Graphics

The Universal Tree of Life

Starting in the 1970s Carl Woese proposed that variation in the sequences of DNA encoding ribosomal RNA (rRNA) in different organisms would provide valuable information regarding evolutionary relatedness. rRNA is an integral part of ribosomal structure, so it is found in all organisms. After comparing the small variations between the genes for rRNA from many organisms, Woese suggested that the Archaea constitute a unique domain of life, a grouping broader than kingdom. The genomes of several members of the Archaea have been entirely sequenced and have been compared with the genomes of other organisms. Such studies confirm that Archaea constitute a separate group: These organisms contain hundreds of genes with no counterparts in Bacteria or Eukarya. Unexpectedly, ribosomal proteins from Archaea were found to be more similar to those of Eukarya than to Bacterial ribosomal proteins. So, Archaea and Eukarya seem more closely related than Bacteria and Eukarya. (See the *Evolution and Phylogenetics* unit.)

Can we construct a tree illustrating the relatedness of the three domains, with one common ancestor for all life? Woese and his colleagues have argued — based on phylogenetic methodology and data from several genes — that there is a common ancestor. They further argue that Archaea and Eukarya are more closely related to each other, and that Bacteria diverged from the common ancestor first. (See the *Evolution and Phylogenetics* unit.)

Other biologists have countered that the true universal tree of life may be more complicated than the picture that Woese and his colleagues presented. The complication is **lateral gene transfer**, where individuals exchange genes between one another. Although not generally exhibited in Eukarya, mechanisms for lateral gene transfer (also known as "horizontal gene transfer") are well known in Bacteria. Genes are exchanged between bacterial species by the action of viruses and by conjugation (cell-to-cell contact in which DNA copied from a plasmid or chromosome is transferred to a recipient cell). Under special conditions, some bacteria are known to take up "naked" DNA from the environment.

Lateral gene transfer, if restricted to very similar organisms, would not pose a problem for constructing a universal tree of life. However, there is evidence that genes have been exchanged between very distant organisms. Eukarya acquired mitochondrial and chloroplast DNA from Bacteria. Nuclear genes in eukaryotes seem to be derived from Bacteria as well, not just from Archaea. Genes are also shared between Archaea and Bacteria. Twenty-four percent of the genome of the bacterium *Thermotoga maritima* contains archaen DNA. Similarly, the archaean *Archaeoglobus fulgidus* has numerous bacterial genes. Some scientists believe that a more diverse community of primitive cells gave rise to the three domains and that the notion of a single universal ancestor might be replaced. W. Ford Doolittle (Dalhousie University) has suggested that lateral gene transfer among early organisms has generated a "tree of life" which more closely resembles a shrub — with untreelike links (shared genes) connecting the branches **(Fig. 3)**.

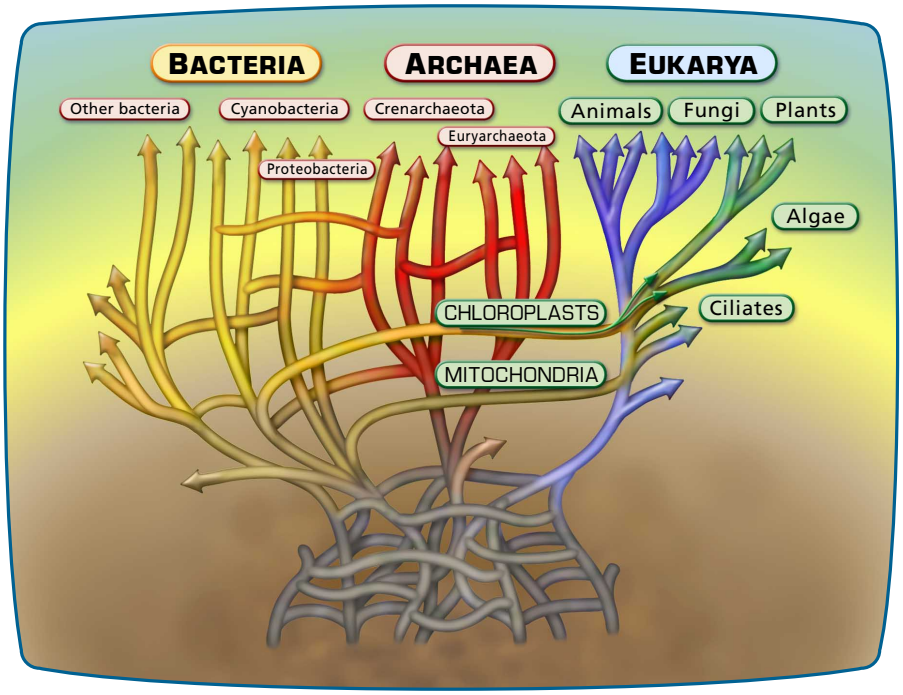


Photo-illustration — Bergmann Graphics

Figure 3. Proposed by W. Ford Doolittle, this view of early evolution suggests multiple primitive cells as ancestors to the three domains, and illustrates lateral gene transfer among early organisms.

Studying Unculturable Microbes with PCR

Imagine yourself on a team studying archaea at a deep-sea hydrothermal vent at the Galapagos Rift (an area known for its hydrothermal activity). You've found a new microbe. What do you want to know about it? What metabolic class does the microbe fall within? Does it make certain proteins? How does it survive the volcanic heat? Traditionally, asking such questions involved growing microbes in the laboratory. Unfortunately, replicating the conditions in which many bacteria and archaea grow is very difficult. For this reason, only a small fraction (perhaps only as few as one percent) of the microorganisms in nature has been cultivated. To identify and compare unculturable organisms microbiologists have turned to molecular genetic techniques.

Polymerase chain reaction (PCR) is one technique for studying organisms that cannot be grown in the laboratory. When only a small quantity of DNA is available from a particular source, PCR can be used to amplify that DNA and produce billions of copies of a designated gene-sized fragment. The technique has many applications, including the amplification of DNA from crime scenes, analysis of cancer genes, and identification of pathogens. When an environmental sample contains unculturable organisms, scientists can use PCR to generate copies of microbial genes suitable for comparison.

To replicate DNA in vitro, PCR takes advantage of a special property of the molecule: the hydrogen bonds. These bonds, which bind the complementary strands of DNA together in a double helix, are broken at elevated temperatures (about 95°C). Each single-stranded piece of DNA (**ssDNA**) is then built upon to form a new, double-stranded molecule (**dsDNA**). To initiate this, short "primers" — specific ssDNA fragments called **oligonucleotides** — must anneal to complementary regions on the single-stranded DNA. Deoxynucleotides (A,T,G, and C) and DNA polymerase are added and, in a process called primer extension, the complementary copy of the ssDNA fragment is built. The

result is two double-stranded DNA molecules identical to the original. Repeating these steps thirty times can result in a 10^9-fold amplification of the original molecule.

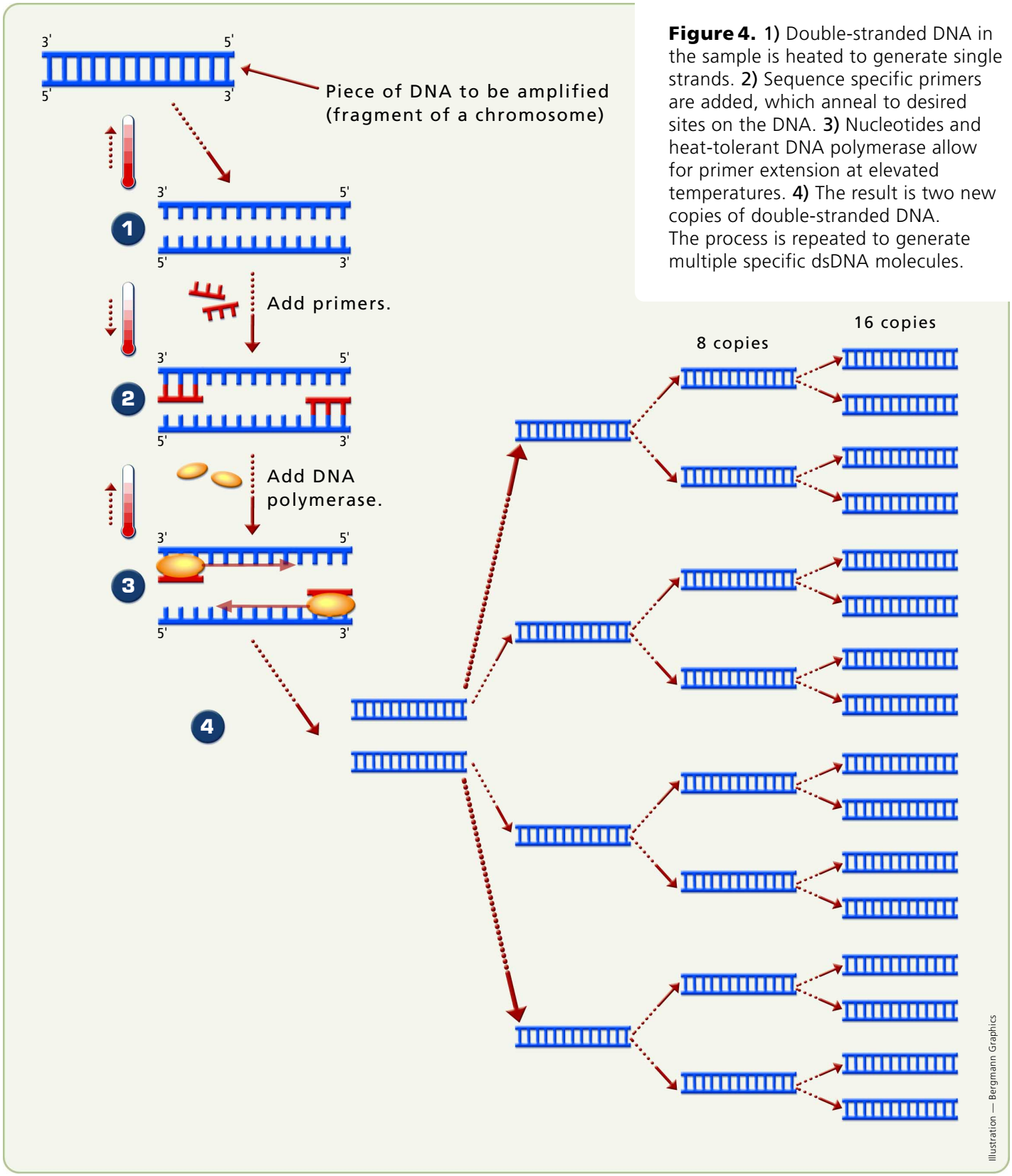


Figure 4. **1)** Double-stranded DNA in the sample is heated to generate single strands. **2)** Sequence specific primers are added, which anneal to desired sites on the DNA. **3)** Nucleotides and heat-tolerant DNA polymerase allow for primer extension at elevated temperatures. **4)** The result is two new copies of double-stranded DNA. The process is repeated to generate multiple specific dsDNA molecules.

Careful thermal cycling is required for PCR to proceed. For the primers to anneal to the ssDNA fragments, the temperature is reduced to about 55°C. However, at this temperature the original complementary ssDNA fragments will begin to re-anneal with each other. A high concentration of primers, and the tendency of the shorter primer strands to anneal more readily, ensures primer binding. The temperature is then raised again to about 72°C for primer extension. Underscoring the importance of microbes, the thermophilic bacteria *Thermus aquaticus* is the major source of the heat-tolerant DNA polymerase, which catalyzes primer extension and facilitates PCR.

In order to amplify a particular gene, specific primers, unique to that gene, are used. Two oligonucleotide primers (oligos) are constructed that flank a region of interest. One oligo will be complementary to a region on one strand of DNA, and the other oligo will be complementary to a region downstream on the homologous strand.

Back home, after your trip to the deep-sea hydrothermal vent, you want to determine what genus of bacteria you have in hand. You can use PCR to amplify the gene for ribosomal RNA (the gene isolated and sequenced by Woese from so many organisms when he constructed his "Tree of Life"). Then, you can choose conserved regions of the rRNA gene for primers. With adequate DNA from PCR, you could sequence the gene and compare it with millions of known rRNA gene sequences using a computer database. (See the *Genomics* unit.)

Alternately, you might want to ask if a microbe carries out a particular form of metabolism. Given the DNA sequence for a protein involved in a particular metabolic strategy — photosynthesis, for example — you could construct oligos so that the presence of that gene could be detected using PCR.

How does your microbe withstand the high temperatures of its volcanic environment? This has been a question posed by researchers studying extreme thermophiles for some time. Indeed, organisms have been found that tolerate temperatures as high as 110°C. Some archaea produce unusually high concentrations of **thermoprotective proteins** (heat shock proteins), that are found in all cells. These proteins help refold partially denatured proteins. Other archaea produce unique proteins that help stabilize DNA. You could use PCR to detect the genes for such proteins in your samples.

As the techniques of molecular genetics are applied to extreme environments we will come closer to understanding the wide variety of strategies that organisms use to survive on this planet... and perhaps on others.

Microbes and the Carbon Cycle

We have classified microorganisms, including archaea, based on their sources of energy and carbon. The cycling of carbon between carbon dioxide and organic compounds is of considerable ecological importance. In addition to eukaryotes (such as plants and algae), autotrophic bacteria (such as cyanobacteria) play an important role in the fixation of carbon dioxide into organic compounds. Consumers, in turn, use organic compounds and release carbon dioxide. Decomposition of plants and animals and their constituent organic compounds is carried out by a large number of bacteria and fungi.

What is taking place in a swamp where you see marsh gas bubbling up though the ooze? A carbon cycle, based on one-carbon compounds, is taking place in the sediments and overlaying water of such freshwater environments. The anoxic sediments harbor archaea, which produce methane as a byproduct of energy metabolism. The methane rises from the sediment and moves into the zone above it. This upper area contains enough oxygen to support methane oxidizers, bacteria that use the methane as a source of carbon as well as an energy source.

Methane (CH_4) is a greenhouse gas and, according to international

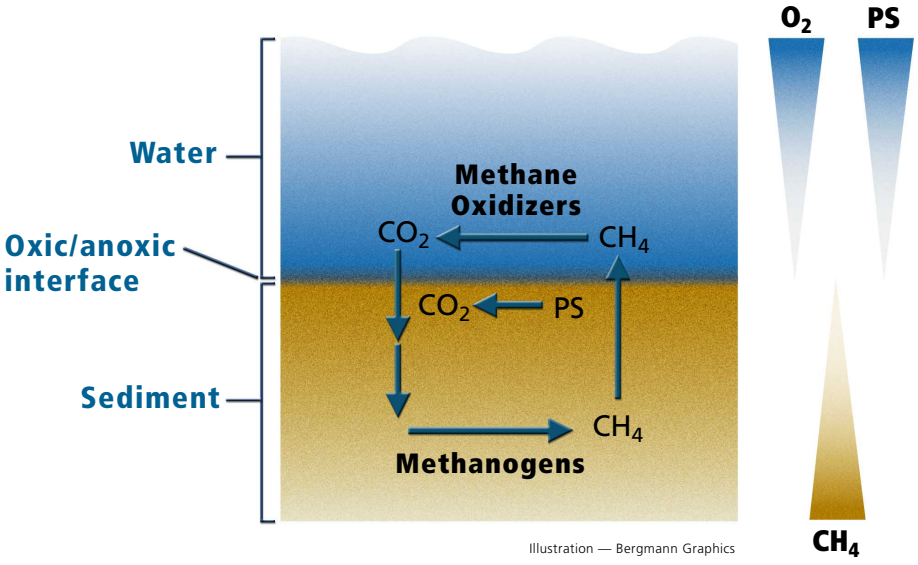


Figure 5. Methanogens are intolerant to oxygen so they thrive in anoxic sediments. The methane they produce is a carbon and an energy source for methane oxidizers in overlaying water.

agreement, its emissions are controlled. Although it is produced by burning fossil fuel, most enters the atmosphere because of microbial action. How can the latter be limited? One strategy is to drain rice paddies more often, limiting the action of methane producers. Another is to add a layer of soil to landfills to encourage methane-oxidizers. Such approaches to reducing this harmful greenhouse gas are under being studied.

Microbes and the Cycling of Nitrogen

Nitrogen is an important part of proteins and nucleic acids. This vital nutrient is recycled from organic compounds to ammonia, ammonium ions, nitrite, nitrate, and nitrogen gas by a variety of processes, many of which depend on microbes. Different organisms prefer nitrogen in different forms. The accompanying figure illustrates nitrogen cycling **(Fig. 6)**. Note that nitrification (the conversion of ammonium to nitrite and nitrate) in soil is carried out by only two genera of bacteria: *Nitrosomonas* and *Nitrobacter.* Denitrification — the loss of nitrate from soil to form gaseous nitrogen compounds (N_2O, NO, and N_2), — is dependent on other kinds of bacteria.

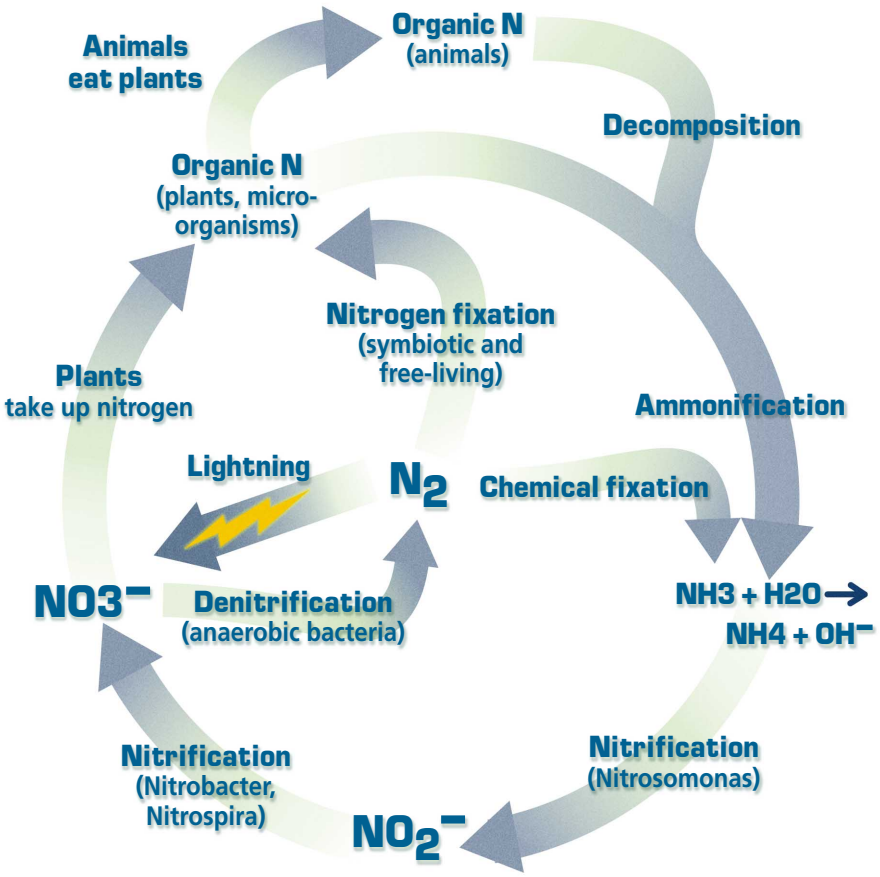


Illustration — Bergmann Graphics

Figure 6. Bacteria are key to the cycling of nitrogen in ecosystems. Different species are involved in decomposition and ammonification, nitrification, denitrification, and nitrogen fixation.

Some prokaryotes are essential to the nitrogen cycle because of their role in nitrogen fixation, the conversion of nitrogen gas to ammonium ions. These ions can then be used to build amino acids. In aquatic environments, cyanobacteria are the most significant nitrogen fixers. In soil, some nitrogen-fixing bacteria are free-living, such as members of the genus *Clostridium;* others live in symbiotic relationship with leguminous plants (such as peas and clover). Symbionts, such as *Rhizobium,* may contribute ten times more nitrogen to soils than free-living bacteria. As we shall see, these symbionts develop intimate relationships with their host plants that require complex communications.

Biofilms

We have formed many of our ideas about bacteria by studying pure cultures — homogenous populations growing in broths. In the wild, however, microorganisms live alongside, in, or on other organisms and often produce proteins not apparent in the laboratory. Bacteria communicate chemically with their neighbors and respond to signals they receive. An understanding of communication among bacteria — including those within bacterial communities — is shaping medical treatments, strategies for bacterial control, and providing a new perspective of the interrelationships between species.

One form of bacterial community is the **biofilm**. An example is the coating of bacteria on your teeth. Biofilms are "living veneers" composed of microcolonies of bacteria, surrounded by a gooey

extracellular matrix that the bacteria secrete. A network of water channels provides nutrients and efficiently removes waste products for the bacteria on the surface. Deeper down, cells rely on diffusion for nutrient delivery and waste removal. Oxygen concentrations vary within a biofilm; cells buried deeper can be oxygen deprived. This variation in environment means that members of a biofilm community, even genetically identical individuals, differ in their metabolic states. In fact, those buried deep within the film are effectively dormant.

"There's a real transformation that takes place and the bacteria start acting like a community... a whole different organism. And there are significant differences in the level of expression of genes within the biofilm because of the different environments within the microcolonies." ANNE CAMPER, CENTER FOR BIOFILM ENGINEERING

Biofilms of *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients can be life threatening. The thick mucus that this inherited disorder produces provides a suitable environment for an infection to become established. However, this is not a simple infection. The bacteria organize themselves into a biofilm and, as they do, some become less susceptible to antibiotics. For patients, the result is a prolonged infection that is very difficult to treat.

Why do bacteria in biofilms survive much higher concentrations of antibiotics and disinfectants than free-living organisms? One reason involves the dormant bacteria in the biofilm. Many antibiotics — penicillin, for example — act only on actively growing cells. Cells that were dormant can serve to reestablish a biofilm once the antimicrobial is no longer present.

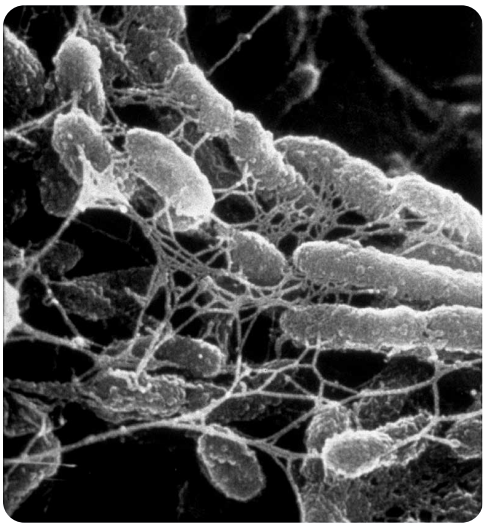
Another mechanism for survival is the layered nature of a biofilm. The effectiveness of a disinfectant, such as bleach, is depleted as it acts on outer layers of the film; bacteria located in inner layers may survive. A third mechanism for survival involves the generation of proteins that provide antimicrobial resistance, such as enzymes that inactivate hydrogen peroxide. Some biofilms are able to manufacture larger quantities of such enzymes so they become more resistant than planktonic (free-floating) bacteria.

Biofilm Formation and Bacterial Communication

How do biofilms form? The formation of a biofilm requires coordinated chemical signaling between cells. Unless an adequate number of neighboring cells are present, the costs of biofilm production to an individual bacterium outweigh the benefits. Thus, a signaling process benefits the bacteria by allowing it to sense the presence of neighboring bacteria and respond to varying conditions. The process by which a bacterium does this is called **quorum sensing**.

Quorum sensing uses signaling molecules, known as **autoinducers**. These are continuously produced by bacteria and can readily diffuse through the cell membrane. When elevated numbers of bacteria are present in an area, the concentration of autoinducers in the region will be higher. Autoinducer molecules (which include certain peptides and

Figure 7. Bacterial cells enmeshed in extracellular matrix material, creating a biofilm.



EXTRACELLULAR MATRIX, W.G. Characklis Collection

compounds known as *homoserine lactones)* can interact with specific repressor or activator sequences in DNA. The presence or absence of the autoinducer thus controls the production of mRNA and, therefore, protein. These proteins are encoded by dozens of genes, including the genes for biofilm production. Laboratory strains of *P. aeruginosa* lacking the gene for a specific homoserine lactone will not develop into normal biofilms but pile up into a disorganized heap.

From the bacteria's perspective, intracellular signaling has many advantages. Often, microbes produce antibiotics that inhibit the growth of competitive species. Intracellular signaling not only brings bacteria together in biofilms, it also regulates the coordinated delivery of high doses of these antibiotics from the denser bacterial population. It also helps bacteria coordinate the release of virulence factors (such as disease-causing toxins) to overcome animal or plant defenses. Signals between bacteria in close proximity, as in a biofilm, also seem to enhance bacterial mating and the acquisition of novel DNA by transformation, both of which increase bacterial diversity.

Impact of Biofilms on Humans

What is the impact of biofilms on humans? Most are benign, like the slippery coating on a rock in a stream, but others can cause serious problems. For example, biofilms contribute to corrosion in metal piping and can reduce the flow of fluids necessary for many industrial applications, including power generation. A particular concern is the contamination of medical devices such as urinary catheters, hemodialysis equipment, and medical and dental implants. Biofilms that develop on these devices can increase the risk of patient infection. The recognition that biofilm formation contributes to disease extends beyond the *Pseudomonas* infections suffered by cystic fibrosis patients. Tuberculosis, Legionnaire's disease, periodontal disease, and some infections of the middle ear are just a few examples of diseases that involve the formation of biofilms. The Centers for Disease Control and Prevention estimates that biofilms account for two-thirds of the bacterial infections that physicians encounter.

Several strategies can be used for attacking biofilms. For example, one might interfere with the synthesis of the extracellular matrix that holds the film together. Scientists are investigating coating medical devices with chemicals that inhibit matrix formation. Another strategy involves inhibiting the adherence of biofilm cells to their substrate. Identifying chemicals that bind to cell surfaces, stopping the formation biofilms before they begin, is also an ongoing interest of researchers. Targeting the molecules that biofilm bacteria use to communicate is a third tactic.

In 1995 Peter Steinberg of the University of New South Wales, Australia, realized that the fronds of a red algae growing in Botany Bay are rarely covered with biofilms. He determined that the algae produce substituted furanones, chemicals that resemble the acylated homoserine lactones necessary for bacterial communication. Evidently, the furanones bind to bacterial cells, thereby blocking the ability of the cells to receive the signals for quorum sensing. Although these compounds are too toxic for human use, similar compounds are being investigated for inhibiting the *Pseudomonas* biofilms that form in cystic fibrosis patients.

Communication Between Bacteria and Eukaryotes

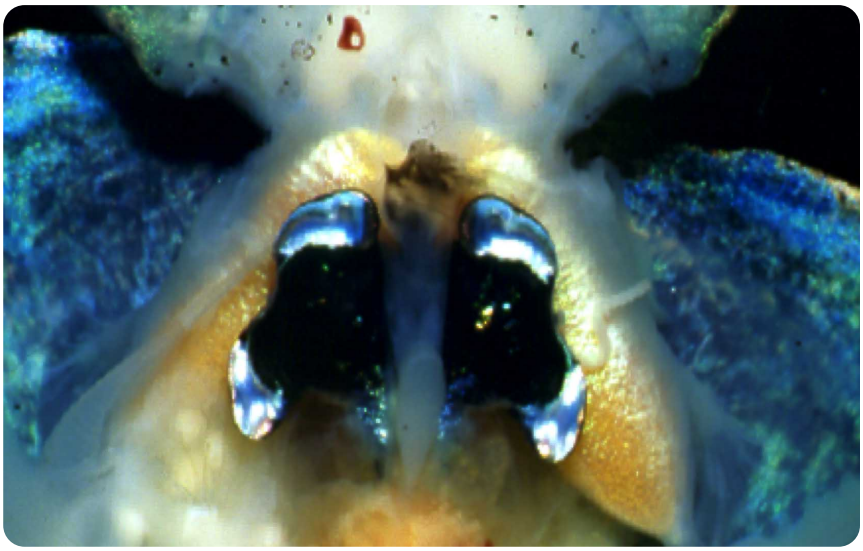
Bacteria also communicate with plants and animals. One striking example involves the *Rhizobium* bacterium which helps fix nitrogen for legumes (such as pea and clover plants). This bacterium colonizes root hairs in specialized nodules built by the plant. Before the plant and bacteria ever come into contact, they are communicating. The plant sends out chemical signals, known as *flavonoids*, which penetrate *Rhizobium* cells and stimulate a gene-activating protein. The protein then switches on bacterial genes so that other proteins, such as Nod factor, are produced. Nod then stimulates the plant to form nodules.

Another example is the signaling between the luminous bacterium, *Vibrio fischeri* and its host, the squid *Eupryman scolopes*. These bacteria colonize a specialized light organ on the squid, providing camouflage. The squid is a nocturnal forager; luminescence from the bacteria erases the shadow that would normally be cast from above by the moon's rays. Quorum sensing molecules allow the bacteria to turn on light production only when the colony has reached adequate density. However, the bacteria do not just communicate with one another — their chemical signals spur maturation of the light organ. Hatchling squid raised in sterile seawater do not develop the pouch which that eventually houses the bacteria.

Like the Dr. Doolittle of fiction, who had the remarkable ability to talk with animals, scientists of the future will be continuing studies into the language of microbes.

Figure 8. The squid *Euprymna scolopes* (left) and its light organ (right). The luminous bacterium *Vibrio fischeri* colonizes the light organ, providing camouflage to the squid.

M. McFall-Ngai and E. Ruby, *EUPRYMNA SCOLOPES.*



M. McFall-Ngai and E. Ruby, *LIGHT ORGAN OF EUPRYMNA SCOLOPES.*

Microbes in Mines

Pyrite (FeS_2), otherwise known as "fool's gold," may not look like lunch to you but it does to the chemolithotrophic bacteria *Acidithiobacillus ferrooxidans* (formerly *Thiobacillus ferrooxidans).* These bacteria extract energy from the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}). Pyrite is one of the most common forms of iron in nature, and is

very common in bituminous coals and in many ore bodies. When pyrite is exposed, as in a mining operation, it reacts with oxygen to generate ferrous ions, sulfate, and hydrogen ions.

$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2Fe^{2+} + 4SO_4^{2-} + 4H^+$

Ferrous ions — lunch! And, the hydrogen ions generated in this reaction do not faze *A. ferrooxidans.* This acidophile prefers a pH below 3.5. It is able maintain a relatively neutral internal pH by actively pumping protons between the cytoplasm and external environment against a steep pH gradient.

Acid mine drainage, which causes serious ecological damage to rivers and lakes, is in part a result of the presence of *A. ferrooxidans.* The ferric ions generated by the bacteria are soluble in the acid environment and easily react with additional pyrite.

$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$

The additional acid that is formed from this reaction is just one of the resultant pollutants. The ferric ions (Fe^{2+}) that are generated precipitate in a complex mineral called jarosite [$HFe_3(SO)_4,(OH)_6$]. The unsightly stains in mine drainages, called "yellow boy" by U.S. miners, are jarosite.

Acid mine drainage and its associated pollution does not form unless pyrite is exposed to oxygen. Only upon mining does the initial reaction generating ferrous ions provide an environment in which *A. ferrooxidans* will thrive.

To reduce toxic metal content in acid mine drainage, scientists are turning to sulfate-reducing bacteria, which occur naturally in anoxic soils. These bacteria use sulfate as an electron acceptor instead of oxygen in a form of metabolism known as **anaerobic respiration**. Hydrogen sulfide is generated in the process. At a bioremediation site in southeast Idaho Dan Kortansky and his colleagues set up a series of ponds separated by berms (embankments) of crushed limestone, straw, and manure. The goal was to convert the sulfate in the drainage to sulfide. This reacts with the dissolved metals to form metal sulfides, such as ferrous sulfide. The limestone in Kortansky's bioremediation system lowers the pH as metal-laden water passes through the berms and sulfate-reducing bacteria thrive. Results have been encouraging. Iron concentrations in the drainage at this site were reduced 65% and copper residues were reduced by nearly 100%.

Microbial Leaching of Ores

Pyrite is not the only mineral oxidized by *A. ferrooxidans* Metals, such as copper, are often present in ores as sulfides. *A. ferrooxidans.* can convert the sulfide chalcolite (Cu_2S) to covellite (CuS) to obtain energy. Copper miners take advantage of this metabolic step during the microbial leaching of low-grade ores. Cu_2S is insoluble but can be converted, by a series of steps (some of which involve the bacteria), to soluble Cu^{2+} ions. Copper metal (C^0) is then recovered when water, rich in copper ions, is passed over metallic iron in a long flume ($Fe^0 + Cu^{2+} \rightarrow Cu^0 + Fe^{2+}$).

In heap leaching, a dilute sulfuric acid solution is percolated through crushed low-grade ore that has been stacked onto an impervious pad.

The liquid coming out of the bottom of the pile, rich in copper ions, is collected and the metal is precipitated by contact with iron (as above). The liquid is then recycled by pumping it back over the pile. Three different oxidation reactions take place within the ore pile:

1. $Cu_2S + O_2 \rightarrow CuS + Cu^{2+} + H_20$ is accomplished by bacteria
2. $CuS + O_2 \rightarrow Cu^{2+} + SO_4^{2-}$ is accomplished by both chemical and biological processes
3. $CuS + 8Fe^{3+} + 4H_2O \rightarrow Cu^{2+} + 8Fe^{2+} + SO_4^{2-} + 8H^+$ is a chemical reaction

The resultant Cu^{2+} is recovered from the solution when it reacts with iron and the Fe^{2+}, which enters the solution, is oxidized (again by *A. ferrooxidans)* to Fe^{3+}. After oxidation this solution is delivered once again to the ore heap. The last oxidation, dependent on the bacteria, provides the Fe^{3+} that drives step 3.

The mining industry has increased biological leaching techniques for various reasons, including environmental concerns related to smelting, the decline in the quality of ore reserves, and difficulties in processing. This new interest has motivated increased research. We now know that ore heaps contain a much wider range of organisms than previously thought. In fact, there is a succession of microbial populations that occurs during the leaching of sulfide minerals. Heterotrophic acidophiles belonging to the genera *Acidiphilium* and *Acidocella* are found frequently, often in close association with *A. ferrooxidans.* These heterotrophic species probably scavenge organic molecules that are metabolic byproducts of the chemolithotrophs. Perhaps this association is detrimental, or perhaps it helps *A. ferrooxidans* thrive by removing wastes.

Research continues into the composition of bacterial communities that occur naturally in bioleaching activities. Because ore heaps get quite hot during bioleaching, scientists are also asking whether novel bacteria — perhaps thermophiles from Yellowstone or deep-sea vents — might be seeded onto heaps to provide more efficient biomining.

Coda

There are about 5,000 known species of prokaryotes, but scientists estimate that true diversity could range between 400,000 and 4 million species. Each has adapted to its particular environment and each performs many roles. Some of these roles are essential to sustaining entire ecosystems. But what is a prokaryotic species? Microbes, which reproduce asexually, cannot be thought of in terms of reproductive isolation. The advent of molecular genetics has brought with it new approaches to defining the concept of species. Some bacteriologists are differentiating prokaryotic species based on their rRNA sequences. If organisms possess rRNA sequences that differ by more than a certain proportion (usually three percent), these bacteriologists consider them to be different species. As new molecular genetic approaches to the study of microbes are developed, scientists will find additional ways of describing the vast diversity of organisms that make up a parallel, albeit invisible, part of our world.

Further Reading

Book

Madigan, M. T., J. M. Martinko, and J. Parker. 2000. *Brock biology of microorganisms,* 9th Ed., Upper Saddle River, NJ: Prentice Hall.
A college-level microbiology textbook with extensive information on Archaea and a clear presentation of microbial mining.

Salyers, A. A. and D. D. Whitt. 2001. *Microbiology — Diversity, disease and the environment.* Bethesda, Maryland: Fitzgerald Science Press.
A college-level microbiology textbook with an ecological emphasis.

Articles

Bassler, B. L. 2001. Tiny conspiracies. *Natural History* 110:16–21.
A review of intracellular communication between microbes. Highlights interactions between the fluorescent bacterium Vibrio fischeri and its squid host.

Costerton, J.W., and Philip S. Stewart. 2001. Battling biofilms. *Scientific American* 285:3–9.
Reviews biofilms. Highlights approaches for inhibiting biofilm formation.

Doolittle, W. F. 2000. Uprooting the tree of life. *Scientific American* 282:90–95.
Introduces alternate systems for describing bacterial relatedness.

Losick, R., and D. Kaiser. 1997. Why and how bacteria communicate. *Scientific American* 276:68–73.
A clear review of bacterial communication.

Reysenbach, A.L., and E. Shock. 2002. Merging genomes with geochemistry in hydrothermal ecosystems. *Science* 296:1077–82.
A review of findings on bacterial populations in hydrothermal vents and volcanic hotsprings.

Glossary

Anaerobic respiration. A pathway of energy metabolism in which an alternate electron acceptor replaces oxygen. Organisms undergoing anaerobic respiration reduce nitrite (NO^{2-}), nitrate (NO^{3-}), or carbon dioxide (CO_2) instead of oxygen.

Archaea. A domain of prokaryotic organisms that differ from bacteria. In contrast to bacteria, archaea lack cell wall peptidoglycan, contain histone-like proteins, and possess chemically distinct cell membrane phospholipids.

Autoinducers. Molecules involved in quorum sensing that regulate mRNA production for specific genes in response to population density.

Biofilm. A multilayered bacterial population embedded in a polysaccharide matrix and attached to some surface.

Chemolithoautotroph. An organism that obtains energy from inorganic compounds and carbon from CO_2.

Chemoorganotroph. An organism that obtains energy from the oxidation of organic compounds.

dsDNA. Double-stranded DNA. A DNA molecule in which two chains (backbones of alternating sugars and phosphates) are linked together by hydrogen bonding between complementary bases.

Eukarya. The domain of all eukaryotic organisms. Eukaryotes are single or multicellular organisms with cells that have a membrane-enclosed nucleus and usually other organelles.

Extremophiles. Organisms that thrive in what humans consider extreme conditions — very salty, hot, cold, acidic, or basic conditions — or at high pressure (such as in the depths of the sea).

Lateral gene transfer. Also referred to as *horizontal gene transfer.* The transmission of genes directly between organisms, particularly bacteria, and not from parent to offspring.

Methanogenic. Methane producing.

Oligonucleotide (oligo). A short, single-stranded DNA molecule consisting of a defined sequence of nucleotides. Used to initiate DNA replication in PCR.

Polymerase chain reaction (PCR). A technique that uses DNA polymerase to amplify the amount of DNA in a sample.

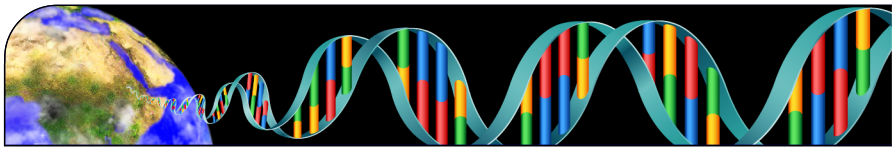
Quorum sensing. A process by which a bacterium detects the density of other bacteria in an area.

ssDNA. Single-stranded DNA. A DNA molecule consisting of only one chain of alternating sugars (deoxyribose) and phosphates.

Stromatolites. Fossilized microbial mats consisting of layers of filamentous prokaryotes and trapped sediment.

Thermophile. Organisms that grow optimally above 45°C. Hyperthermophiles grow optimally above 80°C.

Thermoprotective proteins. Proteins that help bacteria survive heat. Some thermoprotective proteins help refold partially denatured proteins. Others help stabilize DNA.



REDISCOVERING BIOLOGY

Molecular to Global Perspectives

Emerging Infectious Diseases

"To comprehend the interactions between Homo sapiens and the vast and diverse microbial world, perspectives must be forged that meld such disparate fields such as medicine, environmentalism, public health, basic ecology, primate biology, human behavior, economic development, cultural anthropology, human rights law, entomology, parasitology, virology, bacteriology, evolutionary biology, and epidemology." L. Garrett[1]

During the mid-1900s, most scientists and policy makers were shifting their attention away from infectious disease as vaccines made polio and several other diseases rare, at least in the developed world. Through an intense vaccination campaign, researchers at the World Health Organization (WHO) had eradicated smallpox from the world by the mid-1970s. Most people expected that the eradication of other diseases would follow. In the meantime, scientists had created a large array of antibiotics that could easily treat many of the great scourges of history, from leprosy to tuberculosis. Infectious diseases appeared to be on the way out.

This optimistic picture has since changed. Legionnaire's disease, hantavirus, AIDS (acquired immunodeficiency syndrome), West Nile virus, and SARS (severe acute respiratory syndrome) have rocked the public health and scientific communities. New, drug-resistant strains of bacteria have appeared. Tuberculosis and other old diseases, once thought contained, are again a public health concern. In some of these cases the disease-causing agent was previously undescribed. For others a previously treatable pathogen somehow changed. In addition completely new threats emerged. Where had these new threats come from?

Why Do Diseases Emerge?

Many factors contribute to the emergence of disease; outbreaks of existing diseases or the emergence of new ones typically involve several of these factors acting simultaneously. Predicting and controlling emerging infection ultimately requires coming to terms with biocomplexity — the elaborate interrelationships between biological systems (including human social systems) and their physical environments.

Table1. **Factors that affect the emergence of disease** (Smolinski, et al.)[2]
Human behavior and demographics
Microbial adaptation and change
International travel and commerce
Human susceptibility to infection
Technology and industry
Changing ecosystems
Climate and weather
Breakdown of public health measures
Poverty and social inequality
Economic development and land use
War and famine
Lack of political will
Intent to harm

The Human Body as an Ecosystem

The human body is inhabited by billions of bacteria. In fact, we normally carry ten times more prokaryotic than eukaryotic cells. Our mouths alone are host to four hundred identified — and probably hundreds more unidentified — species of bacteria. Most bacteria are benign to their host, some even provide valuable services. For example, bacteria in the gut aid digestion and generate vitamins used by their human hosts.

The bacteria we possess are an ecological community; thus, the principles of community ecology and evolution are vital in understanding how these bacteria (both the benign and the potentially harmful) live within us. Each bacterial species is adapted to the habitat and ecological niche it fills, existing in somewhat of an ecological balance. This balance helps thwart the invasion of pathogens, which must compete with resident bacteria for nutrients and space. Resident bacteria also produce antimicrobial proteins called **bacteriocins**, which inhibit the growth of related species.

When the normal flora are disrupted, it shifts the mix of microbiota and can lead to disease. For example, the use of some broad-spectrum antibiotics can dramatically decrease the numbers of bacteria in the colon. In this situation *Clostridium difficile,* normally present only in low numbers, can overgrow. This bacterium produces toxins that cause potentially fatal damage to the lining of the colon. In the few individuals that normally harbor the microbe, normal levels of other bacteria keep *C. difficile* numbers low. It is only when the balance is disrupted that such a "superinfection" occurs.

The Emergence of Antibiotic-Resistant Bacteria

Today we face a growing medical crisis: the emergence of bacteria resistant to multiple antibiotics. Strains of at least three potentially fatal bacterial species are now resistant to all the drugs available for treatment. *Enterococcus faecalis* is generally a benign intestinal bacterium. In the elderly and individuals with compromised immune systems, however, it can be deadly if it gets in the wrong location. *E. faecalis* can infect heart valves and other organs, causing a deadly systemic disease. Strains of *Pseudomonas aeruginosa* (which causes skin infections and deadly **septicemia**) and *Mycobacterium tuberculosis* (the causative agent of tuberculosis) also evade available drugs. Death rates for tuberculosis have begun to rise, in part because of the evolution of these new strains.

The wide use, and misuse, of antibiotics has encouraged new strains of pathogens to develop. For example, the widespread use of cephalosporin antibiotics has led to drug-resistant *E. faecalis.* The use of Vancomycin (a drug of last resort) has contributed to the development of VRE (vancomycin-resistant *Enterococcus*), which defies treatment. Antibiotic-resistant bacteria are generally not more potent and do not generate a more severe disease state; they are, however, more difficult to treat. Resistant bacteria proliferate when a population of microbes containing both susceptible and resistant bacteria are exposed to an antibiotic within the host: susceptible bacteria succumb and resistant bacteria proliferate.

Public health officials urge people to complete the full course of antibiotic treatment. Why? Bacterial susceptibility to an antibiotic is often dose-dependent; an individual bacterium that is only somewhat resistant may survive at low drug concentrations. There will be selection for such more-resistant bacteria and these will eventually predominate the bacterial population. Thus, the failure of patients to complete a full course of treatment, or the use of less than therapeutic doses of antibiotic, can lead to resistant strains. The full course of treatment should be sufficient to wipe out all the pathogenic bacteria.

An additional cause of the rise of antibiotic resistance is the use of antibiotics in animal feed. Humans may be exposed to such bacteria by handling intestinal contents of the animals, such as when butchering or preparing meats. Moreover, bacteria from livestock can get into our water systems.

Mechanisms of Resistance

Various adaptations provide bacteria with antibiotic resistance. Mutations in a target protein that affect binding of an antibiotic to that protein may confer resistance. If an antibiotic inhibits a metabolic pathway and an alternate one becomes available, resistance can occur. Some antibiotic-resistant bacteria make enzymes that destroy drugs; others alter pores in the cell membrane so an antibiotic can no longer enter. Some resistant strains have developed mechanisms for actively pumping antibiotics out of the bacterial cell. The genes for antibiotic resistance are sometimes found on plasmids. The transfer of these plasmids among bacteria facilitates the spread of antibiotic resistance within and between bacterial populations.

Microbial Adaptation and Change

The evolution of novel microbes, including antibiotic-resistant strains, depends on diverse members of microbial populations that can thrive in new conditions. Microbes have incredible abilities to change their genetic make-up and evolve faster than their hosts do. Multiple mechanisms ensure the diversity that allows for expansion.

The production of a single, novel gene product may be the key to bacterial survival; however, several gene products working together sometimes provide the advantage. Mutation generates new genes but, unlike higher eukaryotes, bacteria do not undergo sexual reproduction; the typical bacterium simply grows, replicates its DNA, and divides. Therefore, bacterial reproduction does not provide a mechanism for generating progeny with new combinations of genes. How, then, do bacteria obtain new gene assortments, some of which may provide survival?

Lateral Gene Transfer

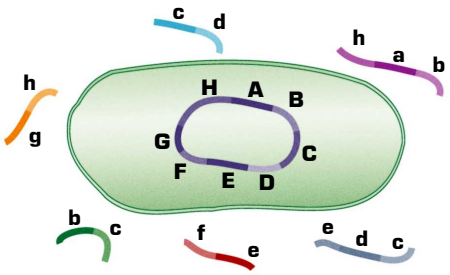
Bacteria possess several methods for **lateral gene transfer** (also called horizontal gene transfer), the transmission of genes between individual cells. These mechanisms not only generate new gene assortments, they also help move genes throughout populations and from species to species. The methods include transformation, transduction, and conjugation.

Transformation involves the uptake of "naked" DNA (DNA not incorporated into structures such as chromosomes) by competent bacterial cells (**Fig. 1**). Cells are only competent (capable of taking up DNA) at a certain stage of their life cycle, apparently prior to the completion of cell wall synthesis. Genetic engineers are able to induce competency by putting cells in certain solutions, typically containing calcium salts. At the entry site, endonucleases cut the DNA into fragments of 7,000–10,000 nucleotides, and the double-stranded DNA separates into single strands. The single-stranded DNA may recombine with the host's chromosome once inside the cell. This recombination replaces the gene in the host with a variant — albeit homologous — gene. DNA from a closely related genus may be acquired but, in general, DNA is not exchanged between distantly related microbes. Not all bacteria can become competent. While transformation occurs in nature, the extent to which it contributes to genetic diversity is not known.

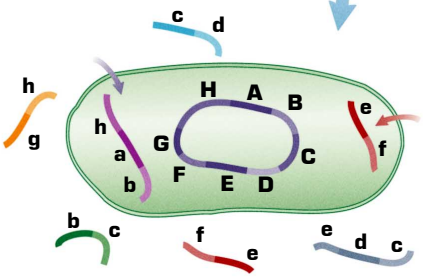
Transduction is another method for transferring genes from one bacterium to another; this time the transfer is mediated by bacteriophages (bacterial viruses, also called phages) (**Fig. 2**). A bacteriophage infection starts when the virus injects its DNA into a bacterial cell. The bacteriophage DNA may then direct the synthesis of new viral components assembled in the bacterium. Bacteriophage DNA is replicated and then packaged within the phage particles. Early in the infective cycle the phage encodes an enzyme that degrades the DNA of the host cell. Some of these fragments of bacterial DNA are packaged within the bacteriophage particles, taking the place of phage DNA. The phage can then break open (lyse) the cell. When released from the infected cell, a phage that contains bacterial genes can continue to infect a new bacterial cell, transferring the bacterial genes. Sometimes genes transferred in this manner become integrated into the genome

Figure 1.
Bacterial Transformation

1. Naked DNA fragments from disintegrated cells in the area of a potential recipient cell. This cell must be of the correct genus and be in a state of competence, allowing the entry of the DNA fragments.



2. Entry of naked DNA into competent cell



3. Recombination

Some DNA Fragments replace (recombine with) original host cell DNA. The resultant recombinant cell will now express the foreign genes it has received and pass them on to all its offspring.

DNA that has not recombined is broken down by enzymes.

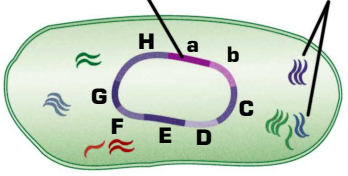


Illustration — Bergmann Graphics

of their new bacterial host by homologous recombination. Such transduced bacteria are not lysed because they do not contain adequate phage DNA for viral synthesis. Transduction occurs in a wide variety of bacteria and is a common mechanism of gene transfer.

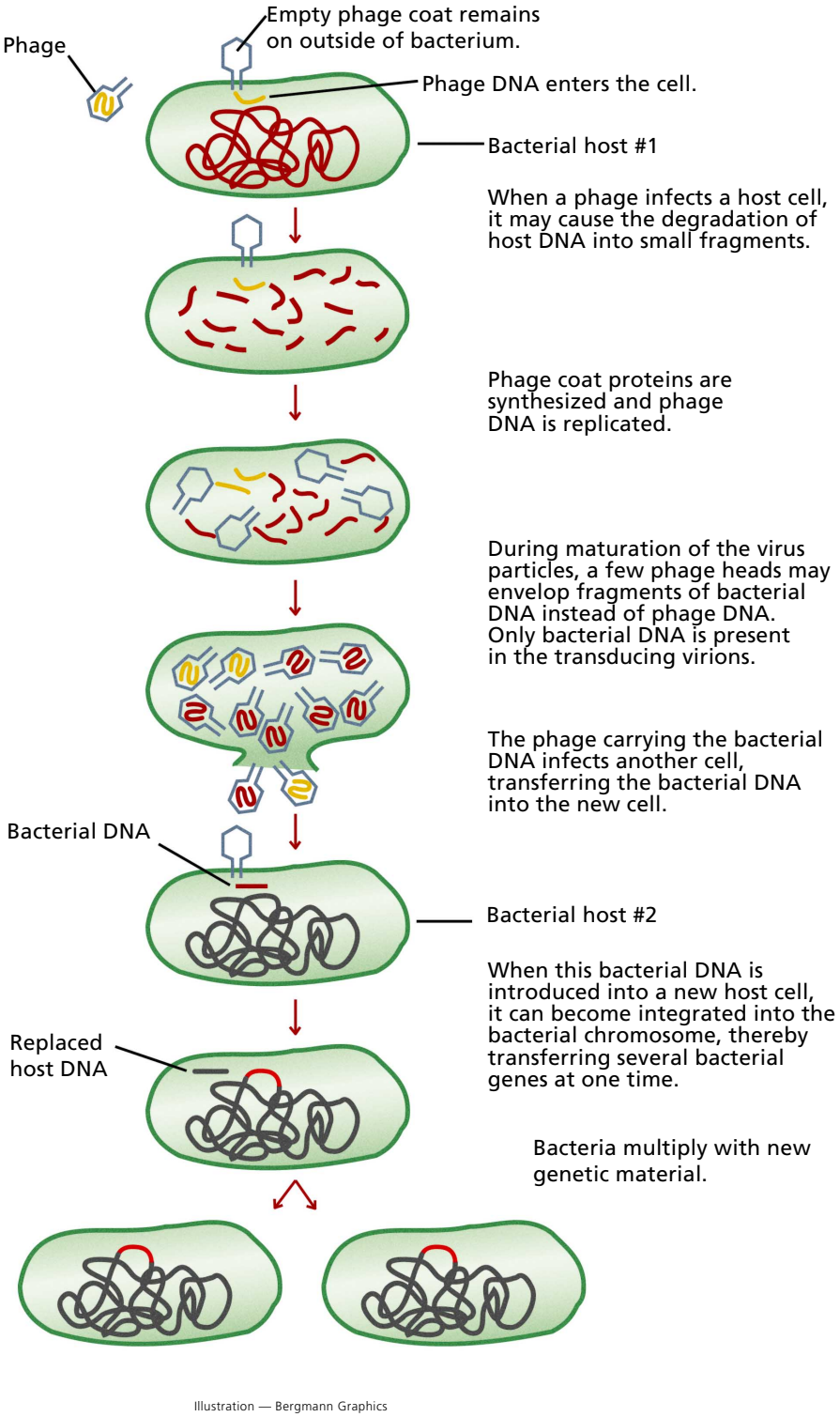
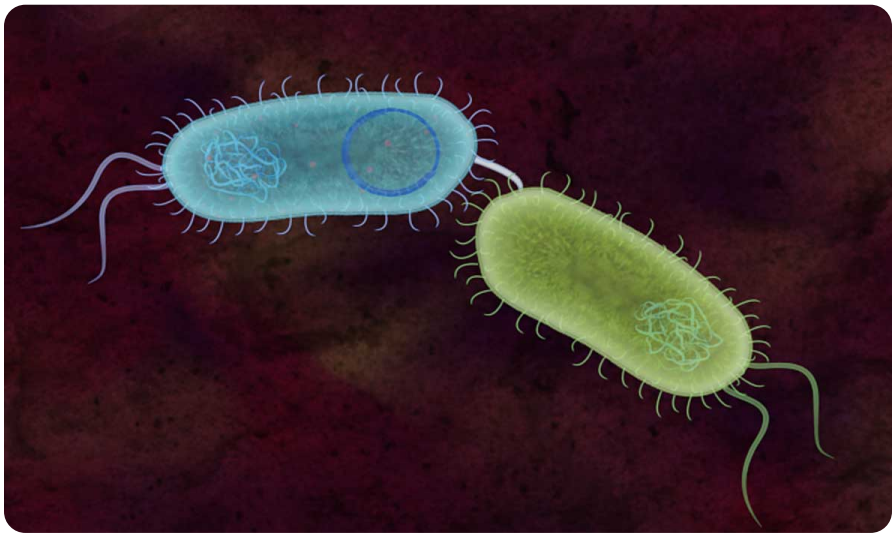


Illustration — Bergmann Graphics

Figure 2.
Transduction by bacteriophage

Some bacteriophages contribute to the virulence of bacterial infections. Certain phages can enter an alternate life cycle called lysogeny. In this cycle, all the virus's DNA becomes integrated into the genome of the host bacterium. The integrated phage, called a prophage, can confer new properties to the bacterium. For example, strains of *Corynebacterium diptheriae*, which have undergone lysogenic conversion, synthesize the toxin in diphtheria that damages human cells. *Clostridium botulinum* and *Streptococcus pyogenes,* when lysogenized by certain phages, also manufacture toxins responsible for illness, causing botulism and scarlet fever respectively. Strains lacking the prophage do not produce the damaging toxins.

Conjugation is another means of gene transfer in many species of bacteria (**Fig. 3**). Cell-to-cell contact by a specialized appendage, known as the F-pilus (or sex pilus), allows a copy of an F- (fertility) plasmid to transfer to a cell that does not contain the plasmid. On rare occasions an **F-plasmid** may become integrated in the chromosome of its bacterial host, generating what is known as an **Hfr** (high frequency of recombination) cell. Such a cell can also direct the synthesis of a sex pilus. As the chromosome of the Hfr cell replicates it may begin to cross the pilus so that plasmid and chromosomal DNA transfers to the recipient cell. Such DNA may recombine with that of its new host, introducing new gene variants. Plasmids encoding antibiotic-resistance genes are passed throughout populations of bacteria, and between multiple species of bacteria by conjugation.



Computer Graphics — Bergmann Graphics & Hotpepper Studios

Figure 3. The F-pilus serves as a point of contact between a bacterium containing an F-plasmid (the "male") and a bacterium lacking the plasmid (the "female"). After the female cell is contacted the pilus retracts, pulling the cells together. The exact mechanism of DNA transfer from male to female is not known; it may be by a channel in the pilus or by a temporary fusion of the mating cells.

Lateral gene transfer is a potent evolutionary force that can create diversity within bacterial species (See the *Microbial Diversity* unit.) As genes for virulence factors and antibiotic resistance spread between and among bacterial populations, scientists are realizing how integral these mechanisms are to the emergence of novel pathogens.

Transposons

Transposons (transposable elements) are genes that can move ("jump") from one DNA molecule to another in a cell, or from one location to another on the same DNA molecule. They can facilitate the transfer of genes, such as antibiotic-resistance genes, from the chromosome of a bacterium to a plasmid. They also can contribute to genetic diversity by causing mutations.

The simplest type of transposon is an insertion sequence (IS). It is a sequence of DNA that encodes an enzyme called transposase, which enables the IS to move. The transposase gene is flanked on either side by fifteen to twenty-five base pairs, arranged as "inverted repeats." A composite transposon is composed of any gene sandwiched between two IS sequences; this entire unit will move.

Travel, Demographics, and Susceptibility

Bacteria move readily from person to person; global travel has contributed significantly to the dissemination of novel pathogens, including drug-resistant strains. Stuart Levy refers to antibiotics as "societal drugs." They not only affect the bacteria in a treated individual, but also produce long-lasting changes in the kinds and proportions of bacteria in the environment and in human populations at large. For example, the multidrug resistant *Streptococcus pneumonia* (a bacterium that causes pneumonia and meningitis) has migrated from Spain to the United Kingdom, the United States, and South Africa.

Crowding also contributes to the dissemination of novel pathogens. Hospitals and nursing homes are particularly ideal environments for the exchange of microbes, including drug-resistant strains. Every year two million people acquire infections while hospitalized and 77,000 people die. Healthy caregivers and visitors can be unwitting carriers, but the scenario is worsened by the compromised status of patients. Cancer treatments and other immunosuppressives, such as those used for transplant patients, contribute to the problem. So does HIV. But any procedure, such as surgery or catheterization, that breaches the protective barrier of the skin increases the risk of infection. In crowded cities, especially in third world countries where adequate sanitation may be lacking, microbes arrive by immigrants from diverse locations. These bacteria can spread rapidly, particularly when immunizations and health care are unavailable.

New Technologies

The evolution of new pathogens is not just a function of human-pathogen or human-human interactions. Sometimes people also unwittingly provide new environments where disease-causing organisms thrive. In the 1970s, for example, air-conditioning systems became widely available. A bacterium normally found in fresh water lakes, *Legionella pneumophila,* moved into the systems, gaining access to susceptible humans. The result was a previously unreported respiratory infection.

Animal Reservoirs

Scientists have identified more than one hundred species of pathogenic bacteria that can infect both humans and animals. As you might imagine, **zoonoses** (diseases that can be transmitted to humans from other vertebrate hosts) are harder to eradicate. For example, Lyme disease is a zoonosis that has emerged, in part, because of human alteration of ecosystems. (See the *Biodiversity* unit.) A recent example of a probable zoonosis is SARS, which has been found in the civet cat and other animals.

Influenza

An average of about 36,000 Americans die each year as a result of influenza. The "Spanish Flu" of 1918–9 killed more people worldwide than did World War I. This disease involves the interaction of multiple animal hosts; however, the story is more complicated. Variation among influenza viruses occurs at the level of the hemagglutinin (HA) and neuraminidase (NA) spikes, which cover the viruses' outer envelopes. These proteins are important for the attachment, and eventual release, of the virus from host cells. In response to an infection the immune system mounts a response against these proteins. Nonetheless, an individual immune to one subtype of influenza may not be able to mount an immune response to a new subtype with modified hemagglutinin or neuraminidase. Genetic mutations, resulting from the change of one or more amino acids within HA or NA, are responsible for the recurrence of minor epidemics of influenza in two to three-year cycles. This is referred to as **antigenic drift.**

More dramatic changes, called **antigenic shifts,** occur when multiple viruses cause coinfections in animal cells (**Fig. 4**). For example, aquatic birds serve as reservoirs for the influenza-A virus. Some, but not all, types of bird influenza can infect humans directly. Occasionally, a new form of the virus — a new human pathogen — arises when multiple viruses infect the same cell. The mixing vessel is often the pig, which can be infected by both the bird and human forms of the virus. Influenza is an RNA virus and its genome is oddly segmented. Genes for HA and NA are found among the eight distinct fragments of single-stranded RNA. If a pig cell is infected with viruses from two different sources, RNA segments might be exchanged. Such genetic exchange can dramatically change the nature of the spikes found on the newly derived virus. Major pandemics of influenza, including the 1918 flu and the "Hong Kong" flu of 1968, have occurred immediately after antigenic shifts have taken place. Farms and markets where poultry, pigs, and humans come in close contact are considered important to the emergence of new subtypes of influenza.

Lyme disease and influenza are just two examples of diseases that have emerged because of human contact with animal reservoirs. Understanding the epidemiology of other emerging infections, such as hantavirus and ebola, also depends on an understanding of animal hosts.

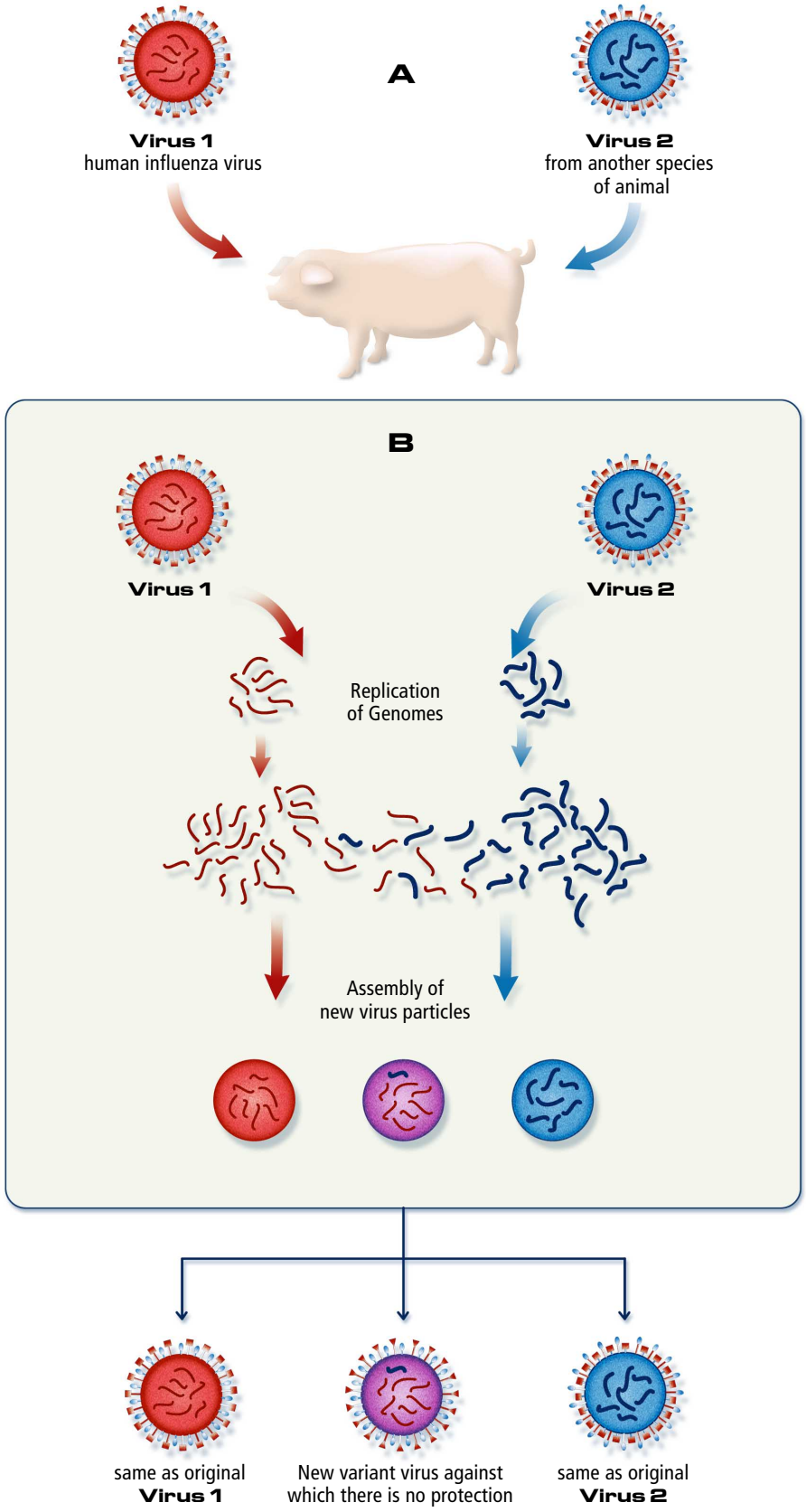


Illustration — Bergmann Graphics

Figure 4. A new form of a virus can arise when multiple viruses infect the same animal cell. Segments of nucleic acid can be exchanged, resulting in a novel pathogen.

Insect Vectors

Insects provide a system that can deliver pathogens directly to the bloodstream and are essential to the spread of some infections. From a pathogen's perspective, moving from host to host is essential to survival; yet, the skin presents a barrier. Wounds, burns, and catheters provide opportunities for entry for some pathogens, but insect-borne bacteria have an advantage. Still, arthropod-transmitted microbes must be able to survive in the arthropod's gut, proliferate, and then become positioned (such as in the insect's salivary gland) for delivery to the animal host.

Malaria

Members of the protozoal species *Plasmodium,* which cause malaria, have evolved a successful relationship with their arthropod vector, the *Anopheles* mosquito. Malaria is prevalent in areas where this mosquito thrives — in parts of Africa, Asia, and China. Three million people die every year from *Plasmodium* infections. Between 1950 and 1970, efforts to eradicate malaria involved the use of the insecticide DDT. Unfortunately, mosquitoes developed resistance to the spray. Now considered a reemerging disease, malaria incidence is on the rise as eradication programs failed and drug-resistant strains of the parasite have evolved. The complex life cycle of the parasite makes development of vaccines difficult, and efforts to reduce malaria by controlling its insect vector continue (**Fig. 5**).

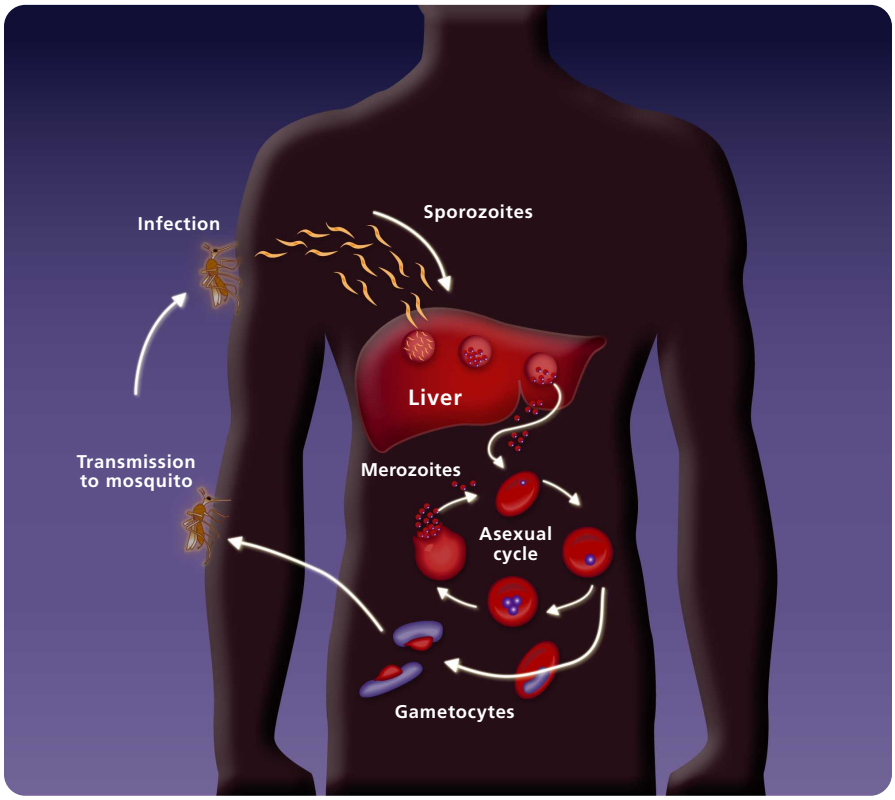


Photo-illustration — Bergmann Graphics

Figure 5. Sporozoites are delivered to the human bloodstream from the salivary gland of the *Anopheles* mosquito when the insect bites. In the liver, the sporozoites multiply and become merozoites. The merozoites enter red blood cells and become trophozoites. Red blood cells rupture and new merozoites, which have developed from the trophozoites, are released. Gametocytes (the sexual stage) are eventually produced. Gametocytes taken up by the mosquito in a blood meal fuse to form zygotes, which give rise to sporozoites.

Dengue

Approximately eighty viruses depend on insects for transmission. The virus that causes dengue and dengue hemorrhagic fever has the broadest distribution, comparable to that of malaria. Approximately 2.5 billion people live in areas at risk for dengue, and millions are afflicted each year. The fatality rate is about five percent, with most fatalities occurring in children and young adults. Transmitted by the mosquito *Aedes aegypti,* dengue, or "breakbone fever," causes a range of symptoms: nausea and weakness, severe bone and joint pain, and high fever.

Four immunologically distinct types of the virus exist, so individuals can contract the disease four times during their lifetime. An infection with a second subtype of the virus may result in a severe **hemorrhagic disease**, involving leakage of blood or fluid from mucous membranes. The hemorrhage seems to involve an immune reaction, resulting from sensitization in a previous infection. A global pandemic of dengue began in southeast Asia after World War II. In the 1980s dengue hemorrhagic fever began a second expansion into Asia, with epidemics in Sri Lanka, India, the Maldive Islands, and, in 1994, Pakistan. During the 1980s epidemic dengue arose in China, Taiwan, and Africa. *Aedes aegypti* and an alternate mosquito vector, *Aedes albopictus,* are present in the United States (**Fig. 6**). Two outbreaks of dengue were reported in Texas during the 1980s, which were associated with epidemics in northern Mexico.

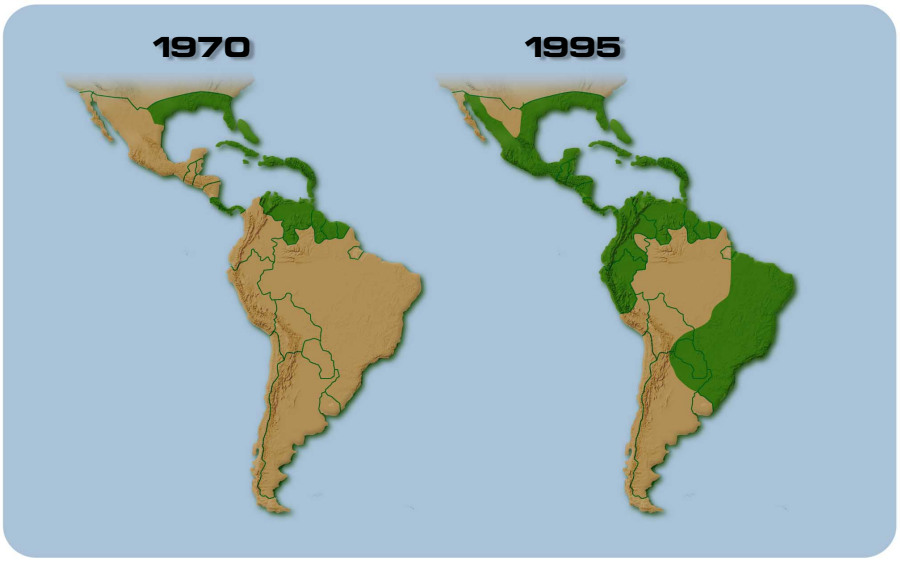


Photo-illustration — Bergmann Graphics

Figure 6. Distribution of the mosquito *Aedes aegypti,* the vector for dengue/dengue hemorrhagic fever. A mosquito eradication program administered by the Pan American Health Organization ended in 1970.

The dramatic global emergence of dengue relates in part to the lack of effective mosquito control in afflicted countries. Often, deteriorating public health infrastructures are to blame.

Climate and Weather

Arthropods, important in the spread of many diseases, are particularly sensitive to meteorological conditions. *Anopheles* mosquitoes, for example, only transmit malaria where temperatures routinely exceed 60°F. Temperature influences the proliferation rate of the mosquito, as well as the maturation rate of the parasite within the insect. Mosquitoes live only a few weeks; warmer temperatures raise the odds that the parasites will mature in time for the insect to spread the protozoans to humans.

Global climate change has already altered the species ranges of a number of animals and plants. (See the *Biodiversity* unit.) Further change may increase the range of the mosquito vectors that transmit disease. This could expose sixty percent of the world's population to malaria-carrying mosquitoes. (Forty-five percent of the human population now reside in a zone of potential malaria transmission.) In fact, malaria is reappearing in areas north and south of the tropics, including the Korean peninsula and areas of Europe. During the 1990s outbreaks of locally transmitted malaria occurred in Texas, Florida, Georgia, Michigan, New Jersey, New York, and Ontario. Although these incidents probably started with a traveler or stowaway mosquito, conditions were such that the infection could be transmitted to individuals who had not been traveling.

Cholera and Global Climate Change

Global climate change may also bring flooding. In addition to creating breeding grounds for insects, this could increase the incidence of water-borne diseases such as cholera. The bacterium *Vibrio cholerae* causes seasonal outbreaks of intestinal infection so severe that individuals can lose as much as twenty-two liters (six gallons) of fluid per day. The intestinal lining becomes shredded so that white flecks, resembling rice grains, are passed in feces. Without adequate fluid replacement, death can occur in hours. During a 1991 epidemic in Bangladesh 200,000 cases were counted in only three months.

Historically, cholera (caused by *V. cholerae*) has been a problem in coastal cities, especially those where the quality of the water supply is poor. In a 1849 groundbreaking study, John Snow mapped cholera deaths in London and realized that victims had been drinking from the same well. The association between cholera and contaminated water was established, and appropriate water treatment seemed to bring the threat under control. Yet, especially in areas where water treatment is unaffordable, cholera epidemics continue.

Where does *Vibrio cholerae* go between epidemics? This question intrigued Rita Colwell and her associates. Surprisingly, they found the bacterium in Chesapeake Bay in a dormant, spore-like form that was difficult to culture in the laboratory. Colwell used antibodies, directed to a component of the bacteria's cell membrane, and was able to detect the dormant organism. In this form *V. cholerae* survives in a range of habitats, including seawater, brackish water, rivers, and estuaries. Colwell also found that wherever tiny crustaceans known as copepods were abundant so were the bacteria, which cling to the copepod and colonize its gut.

Understanding the reservoir for cholera may be important to unraveling the periodicity of epidemics. Colwell turned her attention to locations where cholera outbreaks were common, such as in Bangladesh. By reviewing data from satellite monitors, she noticed that seasonal peaks in sea-surface temperatures in the Bay of Bengal correlated with the number of cholera admissions in nearby hospitals. Similar correlations existed between sea-surface temperatures and South American cholera epidemics in the 1990s. It is possible that the rise in temperature raises sea-surface height, driving seawater into estuaries. Alternately, rising temperatures might provide the right set of environmental conditions to boost copepod populations, perhaps by increasing populations of the photosynthetic plankton, which copepods feed upon. In either case, recognizing the association between sea-surface temperature and cholera incidence may make epidemics easier to predict. The relationship between climate and epidemic also increases the concerns raised by global climate change.

Climate and Hantavirus

Weather patterns can also influence the numbers of vertebrate animals serving as reservoirs for human pathogens. In 1993, in the Four Corners area of the United States (where New Mexico, Arizona, Utah, and Colorado meet), researchers tracked an outbreak of pulmonary illness that killed half of those infected. The causative agent, hantavirus, was not a new threat but was endemic in the rodent population of the area. Researchers were able to find the deadly virus in mouse tissue archived years earlier. Hantavirus spreads to humans by rodent urine and droppings. During the mild, wet winter of 1993, piñon nuts, a favored food for the deer mouse, flourished. As rodent populations soared, the opportunities for mouse-human interactions increased. Native American legend describes an association between piñon nut abundance and illness. Scientists found an association between the periodic climate pattern El Niño-Southern Oscillation and outbreaks of hantavirus.

Medical practices, the adaptability of microbes, global travel, crowding, human susceptibility, alternate vertebrate hosts, insect vectors, and climate are just some of the factors that influence the emergence of disease. In most cases the interplay between multiple factors must be understood. Not the least of these is deteriorating public health systems in many countries where substandard water and waste management continues. War and famine also set up conditions that lead to the emergence of disease and, especially in poor nations, the political impetus to implement prevention and control strategies is often lacking.

Preventing and Controlling Emerging Infectious Disease

The prevention and control of emerging infectious diseases requires a global perspective that accounts for biocomplexity, all the interrelated factors that contribute to the evolution and survival of infectious agents. Individuals from many disciplines — biologists, chemists, statisticians, atmospheric scientists, and ecologists — must work together. Effective surveillance is essential. Multiple control measures will often be appropriate. New genomic and proteomic techniques may provide not only more effective detection but also prevention by novel vaccines.

The effective interaction between public health officials and individuals from a variety of disciplines was exemplified during the West Nile virus outbreak that occurred in the New York City area during the summer of 1999. By mid-October forty-eight people had demonstrated an unusual illness characterized by fever, extreme muscle weakness, and pneumonia-like symptoms. Four had died. Encephalitis or meningitis was present in a few of the more serious cases. West Nile virus was identified as the causative agent using antibody-based tests and DNA comparisons.

West Nile virus hails from Africa, Australia, and the Middle East, and had never been seen in the Western Hemisphere. At the time city wildlife officials and veterinarians at the nearby Bronx zoo were struggling with a peculiar infection among crows and the zoo's collection of exotic birds. Brain hemorrhages and heart lesions were observed in dissected birds; DNA analysis showed the presence of West Nile virus. The discovery of the virus in wild bird populations, which could potentially serve as a reservoir for human disease, spawned surveillance of birds throughout the United States. Concerns that migratory birds would spread the virus rose. Flocks of chickens were used to monitor viral spread. In the meantime New York City began spraying for mosquitoes. By September 2002 the virus had infected a woman in Los Angeles. Continued surveillance of the bird population and continuing communication between wildlife experts, public health workers, and others will be instrumental in curtailing this infection in the United States.

Effective surveillance is a critical step in preventing the spread of emerging diseases. For example, the new influenza vaccine available each year is the result of constant vigilance. The World Health Organization and others identify the strains of influenza most likely to cause infection in the coming year and define the vaccine based on their findings. In this case, an understanding of the animal reservoirs of the disease is important to the surveillance effort. The emergence of novel strains is most likely where poultry, pigs, and humans come in close contact. As a result, monitoring is conducted where such conditions abound.

It is often necessary to take multiple measures to control disease. In the case of malaria the first steps to prevention are as simple as the use of bed nets for reducing bites from mosquitoes and more frequent draining of flooded environments (such as rice fields) where mosquitoes thrive. In the end, DNA-based vaccines, founded on an understanding of the complex life cycle of the protozoal parasite, may be the answer.

Daniel Carucci of the U.S. Naval Medical Research Center and others have identified various proteins that are expressed by the malarial parasite during different stages of its life cycle. Some of these proteins should be recognized as foreign by the immune system and might serve as vaccines. The goal is to stimulate the production of not only antibodies but also cellular immunity specific for various stages of the parasite. (See the *HIV and AIDS* unit for an introduction to the immune system.) Rather than injecting the proteins into individuals, Carucci is evaluating the use of DNA vaccines. Such vaccines usually comprise DNA, encoding the protein(s) of interest, adsorbed onto gold particles and injected with an air gun into muscle tissue. The expression of malarial proteins by recipient cells and the subsequent immune

response to the proteins is being evaluated. If successful, DNA-based vaccines might offer advantages over traditional vaccines; they are less expensive to prepare and easier to store than protein-based vaccines. However, DNA can serve as an immunogen itself; it is thought that diseases such as lupus result from an immune reaction to DNA. As vaccine development continues, the importance of traditional public health measures to prevent and treat malaria remains essential.

The threat from established and evolving disease organisms remains with us. Given high reproductive rates and mechanisms for lateral gene transfer, microbes can adapt to and rapidly circumvent the best treatments scientists develop. We have seen how new diseases arise and spread when humans interact with each other or with the environment in new ways. The anthrax attacks in the fall of 2001 remind us that the threat of bioterrorism continues. This ancient form of warfare dates as far back as 1346 when the Tartar army catapulted the bodies of plague victims into the city of Kaffa.

The journalist Laurie Garrett has suggested that because human behavior influences the emergence of disease, we have significant control over our struggle with microbes. Certainly, our understanding of the factors that contribute to the evolution of new pathogens is continuing to increase, and experience with such outbreaks as West Nile have helped hone surveillance and control measures. However, the global nature of disease means that public health strategies must be global as well.

References

1) Garrett, L. 1994. *The coming plague – Newly emerging diseases in a world out of balance*, 11. New York: Penguin Books.

2) Smolinski, M. S., M. A. Hamburg, and J. Lederberg, eds. 2003. Committee on Emerging Microbial Threats to Health in the 21st Century. *Microbial threats to health: Emergence, detection, and response.* National Academies Press. http://www.nap.edu/catalog/10636.html.

Further Reading

Books

Black, J. G. 2002. *Microbiology – Principles and explorations.* 5th ed. New York: John Wiley and Sons.
A readable college-level microbiology text that focuses on health-related topics.

Garrett, L. 1994. *The coming plague – Newly emerging diseases in a world out of balance*. New York: Penguin Books.
Details the stories of those involved in outbreaks of a variety of emerging diseases, from Ebola and Lassa fever to toxic shock syndrome.

Salyers, A. A., and D. D. Whitt. 2001. *Microbiology – Diversity, disease and the environment*. Bethesda, Maryland: Fitzgerald Science Press
A college-level microbiology textbook with an ecological emphasis.

Articles

Dold, C. 1999. The cholera lesson. *Discover* 20(2):71–75.
Describes Rita Colwell's work on the association between cholera, copepods, and sea surface temperatures.

Epstein, P. R. 2000. Is global warming harmful to health? *Scientific American*, August, 50–54.
Predicts which diseases will be on the rise as global warming progresses.

Gubler, D. J., and G. G. Clark. 1995. Dengue/dengue hemorrhagic fever: The emergence of a global health problem. *Emerg. Infect. Dis.* 1(2):55–57.
Reviews the history and emergence of dengue.

Levy, S. B. 1998. The challenge of antibiotic resistance. *Scientific American*, March, 46–53.
Reviews how and why antibiotic-resistant bacteria arise.

Smolinski, M. S., M. A. Hamburg, and J. Lederberg, eds. 2003. Committee on emerging microbial threats to health in the 21st century. *Microbial threats to health: Emergence, detection, and response*. National Academies Press. http://www.nap.edu/catalog/10636.html.
Presents factors that contribute to the emergence of diseases in great detail.

Webster, R. G. 1998. Influenza: an emerging disease. *Emerg. Infect. Dis.* Vol. 4(3). 436–41.
Reviews the epidemiology of influenza, highlighting the role of bird and animal reservoirs.

Glossary

Antigenic drift. Changes in one or more amino acids in proteins on the outer envelope of a virus such as influenza. Because individuals may not be immune to these modified viruses minor epidemics can result.

Antigenic shift. Changes in proteins in the outer envelope of a virus, resulting from the reassortment of viral genes. Major epidemics of influenza occur after antigenic shifts have taken place because individuals are not immune to the substantially modified viruses.

Bacteriocin. Proteins produced by some bacteria, which inhibit the growth of other strains of the same organism or related species. Genes for bacteriocins may reside on plasmids.

Conjugation. Cell-to-cell contact in which DNA copied from a plasmid or chromosome is transferred to a recipient cell. It can contribute to lateral gene transfer when it occurs between distantly related bacteria.

F-plasmid. A fertility plasmid, which contains genes that allow for conjugation of certain bacteria.

Hemorrhagic disease. Diseases characterized by the leakage of blood or fluid from mucous membranes.

Hfr (high frequency of recombination). A strain of bacteria in which an F-plasmid has become incorporated into the bacterial chromosome.

Lateral gene transfer. Also referred to as horizontal gene transfer. The transmission of genes directly between organisms, particularly bacteria, and not from parent to offspring.

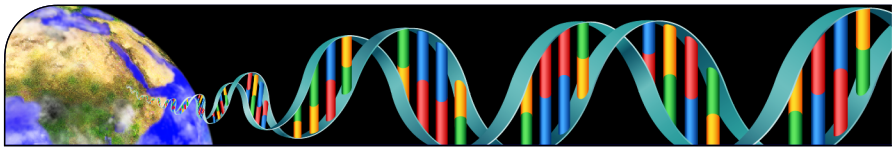
Septicemia. The rapid proliferation of pathogens in the blood.

Transduction. The movement of genetic material from one bacterium to another by means of a bacteriophage.

Transformation. The uptake of "naked" DNA by a bacterium.

Transposon. A DNA sequence that encodes various genes, including those that allow the sequence to jump to other positions within the DNA strand or to other strands of DNA.

Zoonosis. A disease that can be transmitted from other vertebrate animals to humans.



REDISCOVERING BIOLOGY

Molecular to Global Perspectives

HIV and AIDS

"The human immunodeficiency virus (HIV) epidemic has spawned a scientific effort unprecedented in the history of infectious disease research. This effort has merged aspects of clinical research, basic molecular biology, immunology, cell biology, epidemiology, and mathematical modeling in ways that have not been seen before. The ever unfolding discoveries of novel aspects of HIV-host interaction have been accompanied by (and often have resulted from) novel interactions among researchers in the disparate disciplines." John Coffin[1]

In the late 1970s young homosexual men were dying from rare cancers and pneumonias caused by usually benign microbes. Such conditions, which result from failures of the immune system, became indicators of what is now called acquired immunodeficiency syndrome (AIDS). Although the causative virus, human immunodeficiency virus (HIV), was identified in 1983, there is still no cure for AIDS. In the years since, HIV has killed millions of men, women, and children from all economic classes, representing every race, from countries around the world. Each day in 2003, 15,000 more individuals became infected and 8,000 died.

HIV remains a major problem for several reasons. The virus has an extraordinarily high mutation rate, such that an infected individual often harbors many variations. This high mutation rate allows HIV to easily evolve resistance to the drugs used to treat it. In addition, cells essential to a strong immune response harbor a virus that can lay latent for years. Thus, the development of treatments and vaccines depends not only on knowledge of the complex life cycle of the virus, but also on understanding the intricate choreography of the immune system. Controlling HIV will require more than the development of medicines and vaccines, however, because poverty and politics exclude millions from treatment.

The Immune System

Understanding the various components of the immune system and the complex signaling that takes place between immune cells is key to understanding HIV. Both non-specific and specific lines of defense help thwart the invasion of pathogens. Non-specific defenses act quickly and indiscriminately to exclude microbes from the body or actively kill intruders. Mechanical barriers — such as the mucus, hairs, and cilia in the respiratory tract, and the flow of urine through the urinary tract — are among these non-specific defenses. Skin oils and chemicals in

perspiration and gastric juices also serve as non-specific barriers. Mechanisms involving complex chemical signals such as fever and inflammation also act against a wide variety of pathogens. One non-specific defense involves phagocytes, a particular type of leukocyte (white blood cell), which act as cellular "Pac-Men," engulfing and digesting microbes or other irritants like dust and pollen.

If invaders have breached the non-specific defenses, the immune system will use a variety of leukocytes to mount directed defenses against specific invaders. Lymphocytes bind and respond to specific foreign molecules (**antigens**). One subset of lymphocytes, the B cells, matures into antibody-secreting cells. Another subset of lymphocytes, the T cells, includes immune cells that directly kill cancerous or virally infected cells. Some subtypes of T cells serve a regulatory function, releasing chemical signals that can stimulate or suppress a variety of immune functions. Because HIV preferentially infects one of these regulatory T cells, the so-called helper T (T_H) cell, it can subvert and decimate the immune system, leading to AIDS.

Table 1. Types of Leukocytes *(white blood cells)*

Respond non-specifically		
Granulocytes (contain cytoplasmic granules)	**Basophils**	Important in inflammation and allergic responses
	Neutrophils	Phagocytic; during inflammation they squeeze through capillaries to destroy microbes in tissue
	Eosinophils	Phagocytic; elevated in allergy and in parasite infections
Monocytes and macrophages		Monocytes that leave the circulation then mature into highly phagocytic macrophages in tissue. "Fixed" macrophages stay in certain places, such as the lymph nodes or the lung.
Interact with specific antigens		
Lymphocytes	**B cells**	Cells that provide immunity to antigens circulating in the blood, such as bacteria, toxins, and circulating viruses. B cells mature from stem cells in the bone marrow. Once they encounter antigen, B cells mature into plasma cells that secrete antibodies.
	T cells*	Cells that provide cellular immunity to antigens inside or associated with cells, such as cancer cells or cells infected with a virus. They also help clear infections caused by fungi and worms, and contribute to transplant rejection. They mature from stem cells in the thymus. Types of T cells include: • **T_C** - cytotoxic T cells; lyse cells expressing foreign antigens • **T_H** - helper cells; secrete chemicals that enhance T_C and B cell responses • **T_S** - suppressor cells; reduce T_C and B cell responses • **T_D** - delayed hypersensitivity cells involved in certain allergic-like responses

** T cells can be differentiated, in part, based on certain proteins on their surfaces. Helper T cells, which are often called T4 cells, express the CD4 protein. Cytotoxic T cells and suppressor T cells express CD8. Because HIV infects helper T cells, the ratio of CD4 to CD8 cells is valuable for monitoring the course of infection.*

The Central Role of Helper T Cells

Helper T (T_H) cells are critical to coordinating the activity of the immune response. The chemical messages they secrete (**cytokines**) stimulate the non-specific immune response to continue, and strengthen and boost appropriate specific responses. Helper T cells have sometimes been called the "conductors" of the immune system because they coordinate activity like the conductor of a symphony. They have also been called the "generals" of the immune system because they call up troops of B cells, cytotoxic T cells, and other helper T cells to go into battle against invading pathogens (**Fig. 1**).

Macrophages alert helper T cells to the presence of pathogens. These phagocytic macrophages engulf bacteria and viruses, and can display foreign antigens — the identifying proteins of the bacteria or viruses — on the surface of their cell membrane. Embedded within the macrophage cell membrane is a molecule produced by the human leukocyte antigen (HLA) complex. (See the *Human Evolution* unit.) The helper T cells bind simultaneously to the foreign antigen and the HLA molecule. Only T_H cells with receptors that match those of the foreign antigen on the activated macrophage are able to bind and respond to the call to action. Once bound, the helper T cell proliferates to form a clone of cells, each capable of recognizing the same antigen. The members of the helper T clone, the generals, generate the chemical signals that call up the troops.

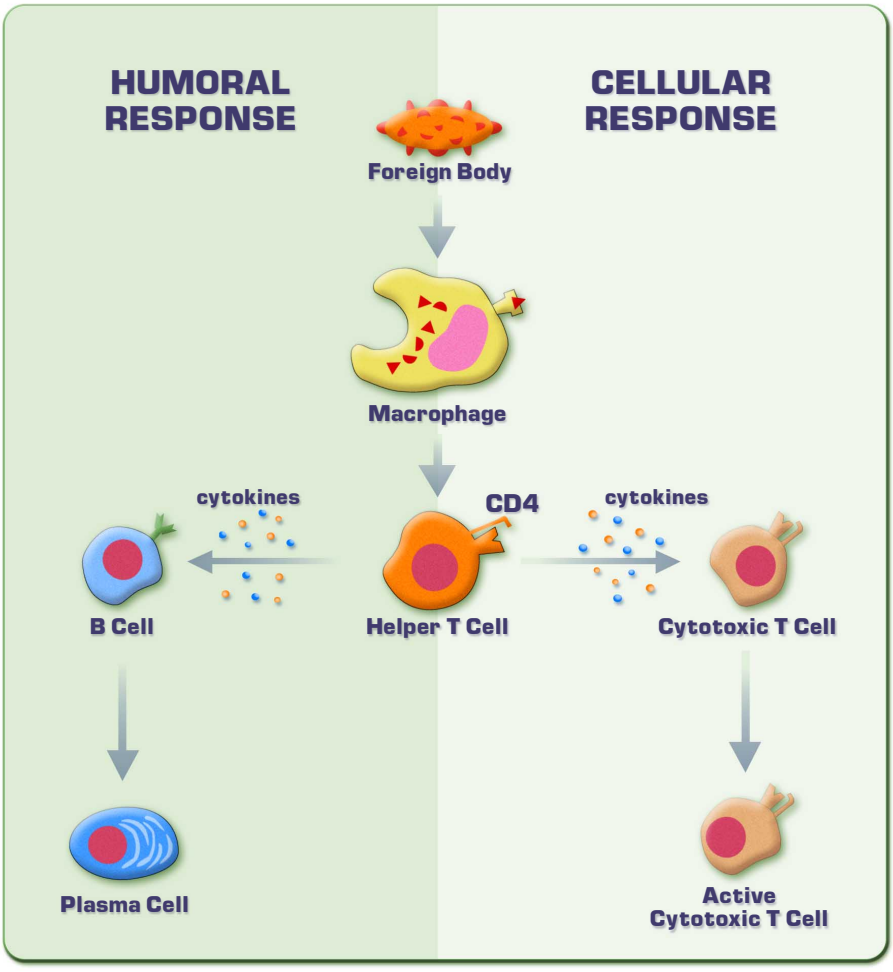


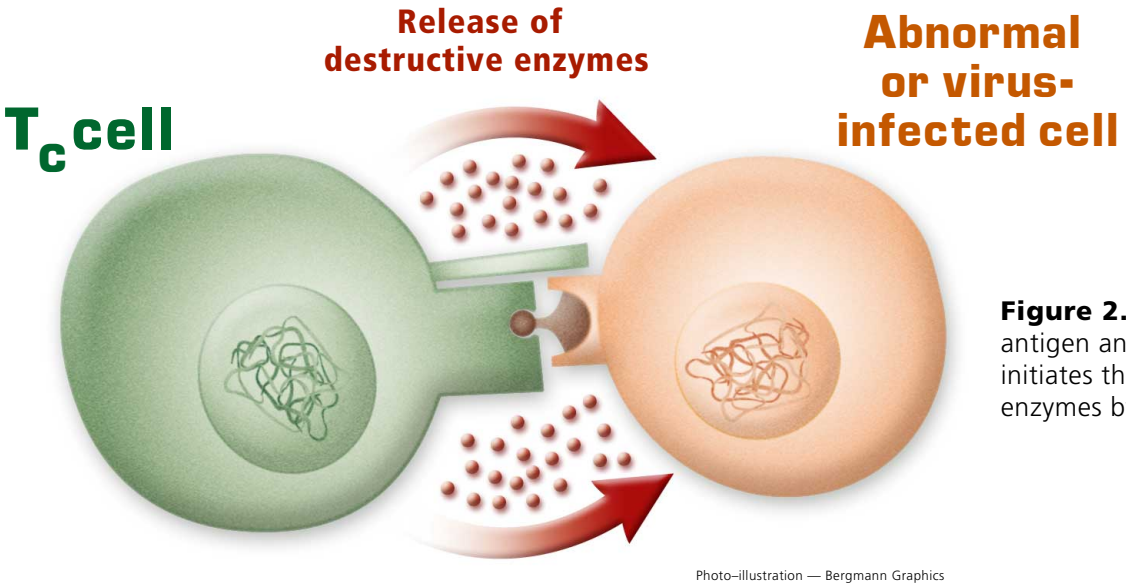
Photo-illustration — Bergmann Graphics

Figure 1. A specialized macrophage ingests foreign antigens and displays antigen fragments along with MHC (self) molecules on its surface. A helper T cell (T_H) with the appropriate receptor binds and responds by producing cytokines that stimulate antigen specific B cells, as well as specific cytotoxic T cells.

Some signals sent by helper T cells stimulate cytotoxic T cells (T_C). Cytotoxic T cells (also known as killer T cells) bind cells that have been altered, such as by viral infection; they avoid healthy cells. Surface antigens on the altered cell perform the binding. These antigens are specific to the offending agent, and match receptors in the membrane of the specific T_C cell. In addition, the T_C cell simultaneously binds an MHC molecule on the surface of the infected cell. Once bound by both the foreign antigen and the HLA molecule, the cytotoxic T cell secretes a chemical called "perforin," which destroys the offending cell (**Fig. 2**).

Helper T cells also stimulate the production of antibodies. Chemical signals from helper T cells stimulate the production of B cells specific to an infecting pathogen, and then stimulate the B cells to differentiate into plasma cells. The plasma cells are factories for the production of antibodies, which are specific to given pathogens circulating in blood or lymph. Antibodies work by blocking the receptors that allow pathogens to attach to target cells, or by creating clumps of bacteria. Clumping makes the job of phagocytes easier, as they will more readily engulf bacteria in clumps. Bound antibodies sometimes serve as tags, called **opsonins**, enhancing phagocytosis. Antibody binding can also initiate a cascade of biochemical reactions, activating a set of chemicals known as complement. Activated complement components can form holes in bacterial membranes and enhance inflammation.

Helper T cells are clearly critical to the operation of the immune system. If they are destroyed because of an HIV infection, the whole system is crippled. The immune system is described as having two "arms": the **cellular** arm, which depends on T cells to mediate attacks on virally infected or cancerous cells; and the **humoral** arm, which depends on antibodies to clear antigens circulating in blood and lymph. As an HIV infection progresses, destroying helper T cells, both arms of immunity are impaired.



Photo–illustration — Bergmann Graphics

Figure 2. Binding by both the antigen and an MHC molecule initiates the secretion of lytic enzymes by the cytotoxic T cell (T_c).

The Structure and Life Cycle of HIV

How does HIV evade the immune system so efficiently? Why are so many variants of the virus found in a single patient? Understanding the structure and life cycle of the virus is key to answering these questions and essential to the design of effective treatments.

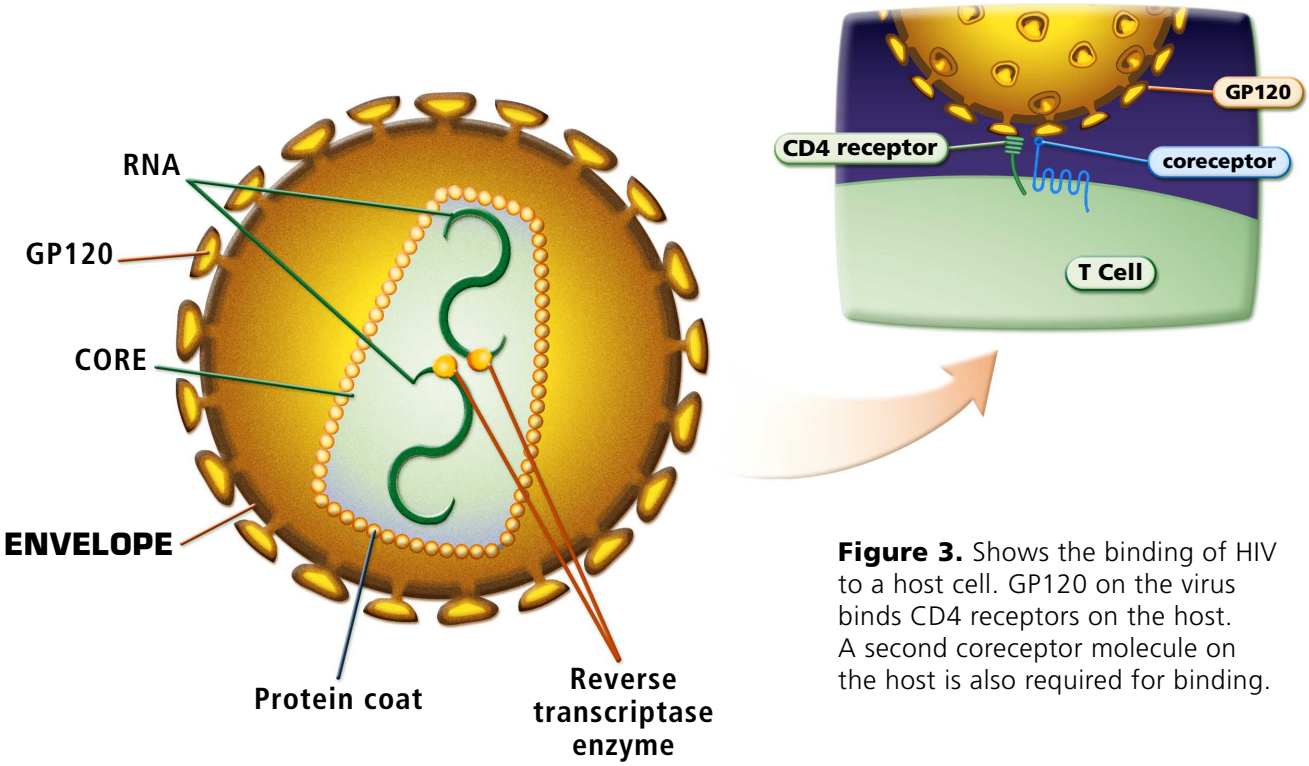


Photo-illustration — Bergmann Graphics

Figure 3. Shows the binding of HIV to a host cell. GP120 on the virus binds CD4 receptors on the host. A second coreceptor molecule on the host is also required for binding.

HIV is an enveloped RNA virus: As HIV buds out of the host cell during replication, it acquires a phospholipid envelope. Protruding from the envelope are peg-like structures that the viral RNA encodes. Each peg consists of three or four gp41 glycoproteins (the stem), capped with three or four **gp120** glycoproteins. Inside the envelope the bullet-shaped nucleocapsid of the virus is composed of protein and surrounds two single strands of RNA. Three enzymes important to the virus's life cycle — **reverse transcriptase**, **integrase**, and **protease** — are also within the nucleocapsid (**Fig. 3**).

Although helper T cells seem to be the main target for HIV, other cells can become infected as well. These include monocytes and macrophages, which can hold large numbers of viruses within themselves without being killed. Some T cells harbor similar reservoirs of the virus.

Entry of HIV into the host cell requires the binding of one or more gp120 molecules on the virus to CD4 molecules on the host cell's surface. Binding to a second receptor is also required. Ed Berger helped identify this coreceptor. As he compared his results with those of other researchers, it became clear that two different coreceptors are involved in the binding. One, CCR5, a **chemokine receptor**, serves as a coreceptor early in an infection. Another chemokine receptor (CXCR4) later serves as a coreceptor. That two coreceptors are involved is consistent with previous observations. Viruses isolated from individuals early in an infection, during the asymptomatic phase, will typically

infect macrophages in the laboratory, but not T cells (the viruses are M-tropic). Virus isolated from patients later in the infection, in the symptomatic phase, will infect T cells (the viruses are T-tropic). It seems that a shift takes place in the viral population during the progression of the infection so that new cellular receptors are used and different cells become infected.

HIV is a member of the group of viruses known as retroviruses, which share a unique life cycle **(Fig. 4)**. Once HIV binds to a host cell, the viral envelope fuses with the cell membrane, and the virus's RNA and enzymes enter the cytoplasm. HIV, like other retroviruses, contains an enzyme called reverse transcriptase. This allows the single-stranded RNA of the virus to be copied and **double-stranded DNA** (**dsDNA**) to be generated. The enzyme integrase then facilitates the integration of this viral DNA into the cellular chromosome. **Provirus** (HIV DNA) is replicated along with the chromosome when the cell divides. The integration of provirus into the host DNA provides the latency that enables the virus to evade host responses so effectively.

Production of viral proteins and RNA takes place when the provirus is transcribed. Viral proteins are then assembled using the host cell's protein-making machinery. The virus's protease enzyme allows for the processing of newly translated polypeptides into the proteins, which are then ultimately assembled into viral particles. The virus eventually buds out of the cell. A cell infected with a retrovirus does not necessarily lyse the cell when viral replication takes place; rather, many viral particles can bud out of a cell over the course of time.

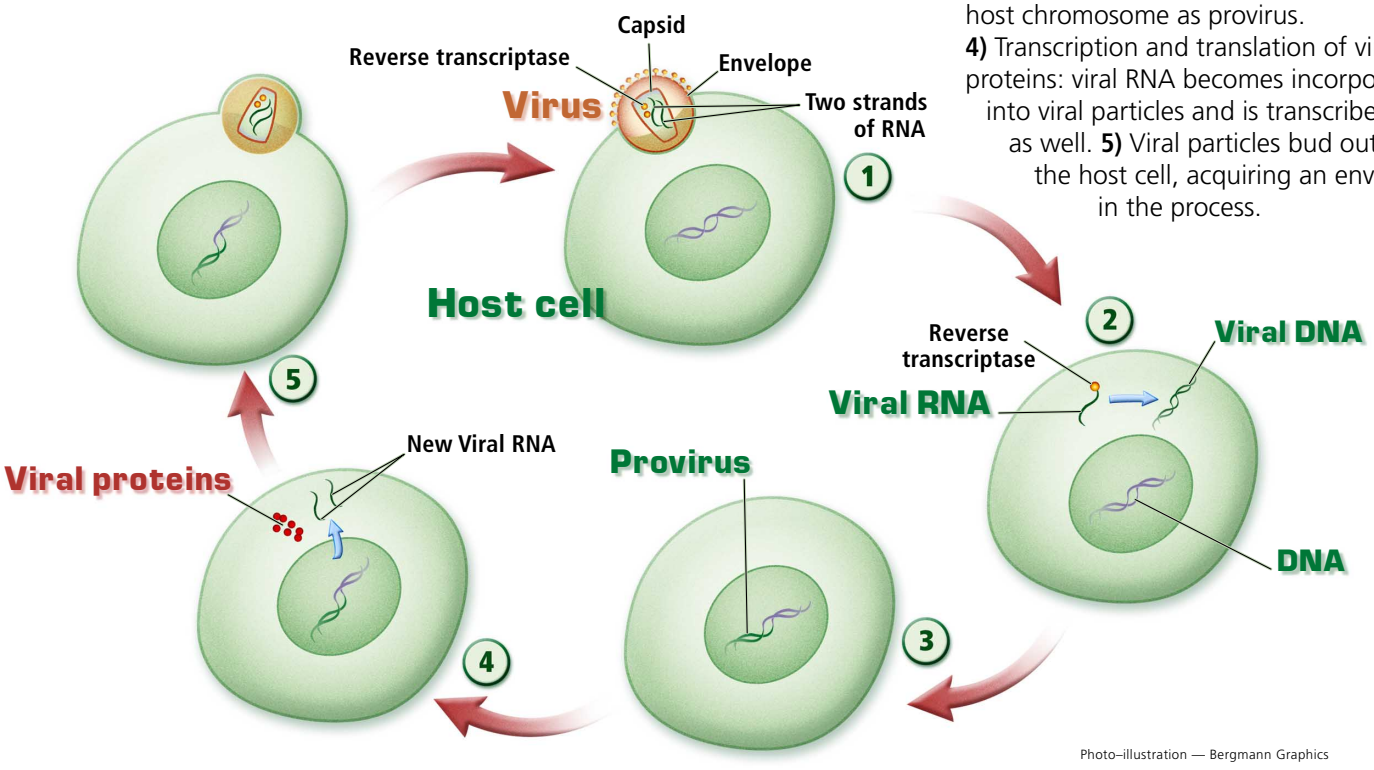


Figure 4. **1)** Membranes of the virus and the host cell fuse, and viral RNA and reverse transcriptase enter the host's cytoplasm. **2)** Reverse transcriptase allows viral RNA to be copied to DNA. **3)** Viral DNA is incorporated into the host chromosome as provirus. **4)** Transcription and translation of viral proteins: viral RNA becomes incorporated into viral particles and is transcribed as well. **5)** Viral particles bud out of the host cell, acquiring an envelope in the process.

HIV Transmission

HIV is transmitted principally in three ways: by sexual contact, by blood (through transfusion, blood products, or contaminated needles), or by passage from mother to child. Although homosexual contact remains a major source of HIV within the United States, "heterosexual transmission is the most important means of HIV spread worldwide today."[2] Treatment of blood products and donor screening has essentially eliminated the risk of HIV from contaminated blood products in developed countries, but its spread continues among intravenous drug users who share needles. In developing countries, contaminated blood and contaminated needles remain important means of infection. Thirteen to thirty-five percent of pregnant women infected with HIV will pass the infection on to their babies; transmission occurs in utero, as well as during birth. Breast milk from infected mothers has been shown to contain high levels of the virus also. HIV is not spread by the fecal-oral route; aerosols; insects; or casual contact, such as sharing household items or hugging. The risk to health care workers is primarily from direct inoculation by needle sticks. Although saliva can contain small quantities of the virus, the virus cannot be spread by kissing.

Progression of HIV Infection

Characteristically, an HIV infection can progress for eight to ten years before the clinical syndrome (AIDS) occurs. The long latent period of the virus has contributed to many of the problems relating to diagnosis and control. The basketball player Magic Johnson was still relatively healthy twelve years after he announced he had HIV. On the other hand, not all cases exhibit the long latent period, and abrupt progression to AIDS occurs. Many factors, including genetics, determine the speed at which the disease will progress in a given individual.

The Centers for Disease Control and Prevention (CDC) has identified the stages of a typical HIV infection: Categories A, B, and C. In the first stage, Category A, it can be difficult to determine whether an individual is infected without performing a blood test. While at least half of infected individuals will develop a mononucleosis-like illness (headache, muscle ache, sore throat, fever, and swollen lymph nodes) within three weeks of exposure, some Category A individuals are asymptomatic. Moreover, the symptoms themselves can be the result of many different infections. The presence of a rash may help differentiate an HIV infection from other infections, but not all HIV-infected individuals get a rash. Most of these signs and symptoms subside, but swollen lymph glands and malaise can persist for years through Category A HIV.

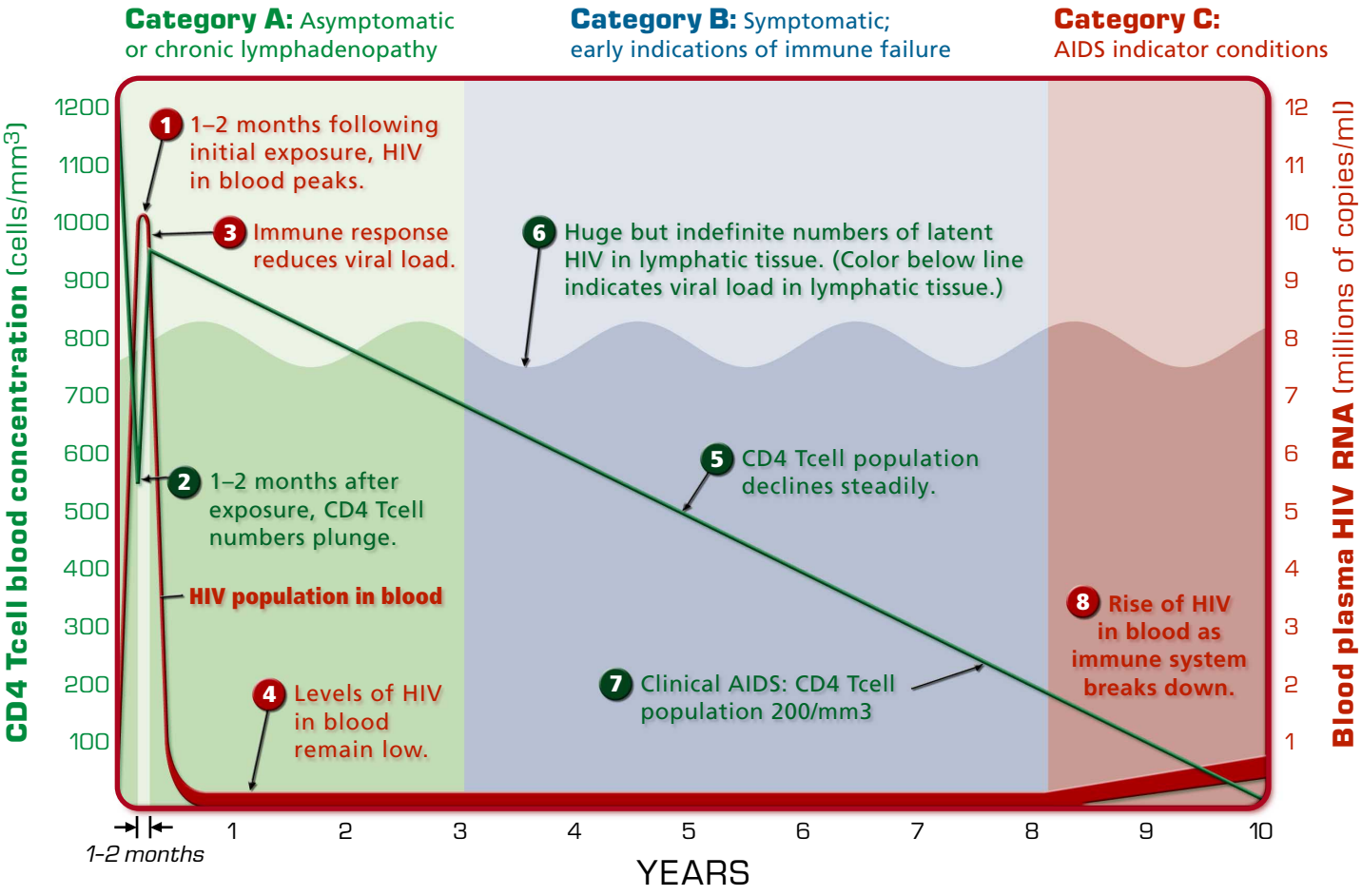


Figure 5.
Typical Progression of HIV Infection & AIDS.

The number of virus particles circulating in the bloodstream is usually highest soon after exposure. At this point the CD4 cell population plunges (helper T cells are among the immune cells that express the CD4 receptor, which can be used as a marker for counting cell types). As antibodies to HIV appear the numbers of CD4 cells rise; however, CD4 cell levels drop again as the infection progresses. This lowering of CD4 cell levels typically happens slowly, over the course of years. Category C HIV (clinical AIDS) occurs once CD4 numbers have fallen substantially (to 200/mm^3 from the normal level of 800–1200 cells/ mm^3).

In the Category B stage indications of immune system failure begin. Persistent infections — such as yeast infections, shingles, diarrhea, and certain cancerous conditions of the cervix — are apparent.

Category C is synonymous with AIDS. In this stage the opportunistic infections associated with AIDS appear. According to the CDC, twenty-six known clinical conditions affect people with AIDS; most are infections that do not usually affect healthy individuals. These include yeast infections of the esophagus, bronchi, and lungs; *Pneumocystis* pneumonia (a fungal infection); toxoplasmosis (caused by a protozoan that is spread by cats); Kaposi's sarcoma (a rare cancer of the skin caused by a virus); cytomegalovirus (CMV) infections; and tuberculosis. In addition, individuals who have been affected by HIV are more likely to become seriously ill or die than other members of the population during outbreaks of infections such as cryptosporidium (a water-borne parasite) and coccidiomycosis (a dust-borne fungus).

Cytomegalovirus (CMV) causes another opportunistic infection prevalent in AIDS patients. About eighty percent of people in the U.S. have antibodies to this virus, but infections in normal individuals often go undetected or seem like a mild case of mononucleosis. In the immunocompromised, however, CMV can cause life-threatening pneumonia or encephalitis. In AIDS patients CMV that has been latent can reactivate and sometimes cause retinitis, affecting eyesight.

Tuberculosis (caused by *Mycobacterium tuberculosis*) has been on the rise in the wake of AIDS, such that some call it a coepidemic. *M. tuberculosis* causes a respiratory infection (formerly called consumption) that is spread by inhalation. As a result, unlike HIV, behavior modification is less likely to reduce one's chances of exposure. The bacteria, which have an unusually waxy cell wall, survive well in the environment. *M. tuberculosis* reproduces inside macrophages found in the lung, and stimulates the production of aggregates of immune cells and connective tissue, called tubercles. Viable organisms can be walled off within such structures for decades, only to become reactivated when a person becomes compromised. Most tuberculosis in AIDS patients results from reactivated infections. AIDS patients suffer not only from respiratory infection but also from disseminated tuberculosis, which can involve the lymphatic system, peritoneum, meninges, urogenital system, or digestive tract. Antibiotic-resistant mycobacteria are also contributing to the rise of tuberculosis, so that second- and third-line drugs must often be used. And because treatments are prolonged, lasting as long as a year, patients sometimes do not complete therapy appropriately. Mycobacteria other than *M. tuberculosis*, particularly *M. avium-intracellulare* (MAC), also affect AIDS patients.

Why Do Some Individuals Never Get AIDS?

Despite repeated exposure, some individuals never become infected with HIV. These individuals often have unusual helper T cells with a less-efficient variant of the coreceptor CCR5, which is necessary for viral entry into helper T cells. (See the *Human Evolution* unit.)

There are also individuals who become infected, but do not progress to AIDS. These long-term survivors, or long-term non-progressors, include individuals who have been AIDS-free as long as eighteen years after infection. A variety of factors may be responsible; for example, infection with less-virulent viruses. Some long-term non-progressors seem to have CD8 cells, which are particularly adept at curtailing HIV infection. (In most AIDS patients CD8 cells become less active.) Several investigators, including Jay Levy (University of California, San Francisco), are evaluating the CD8 cells of long-term survivors to see of they secrete an antiviral protein or proteins that may act against HIV.

Genetic Variation Among HIV

There are five major subtypes of HIV, designated A through E. Different subtypes predominate in different geographical areas. For example, subtype B is more common in North America. In contrast, subtype C predominates in sub-Saharan Africa. Considerable variation within a given subtype also exists. In fact, any given individual infected with HIV will harbor multiple variants of the virus. HIV makes many

mistakes as is copies its viral RNA to the DNA that integrates into the host's chromosome. Because of its sloppy copying of reverse transcriptase, HIV's mutation rate is high, causing great variability. This large number of variants makes the virus more difficult to treat and hinders vaccine development. In addition, because of its rapid rate of evolution, even within a single individual, HIV can quickly evolve resistance to the drugs the individual is taking to combat the virus.

Treatments Based on Understanding the Viral Life Cycle

Treating viruses is always difficult because viruses use the translational machinery of the host cell. Most drugs that target the virus also damage the host. Drugs that can inhibit enzymes specific to the virus are, therefore, less likely to cause side effects in the host.

Most common anti-HIV drugs block key steps in viral reproduction and uptake. Several anti-retroviral drugs work by interfering with reverse transcriptase, the key enzyme of retroviruses. These drugs, the reverse transcriptase inhibitors, act when cells first become infected. Included in this group are the nucleoside analogs, chemicals that are similar to one of the bases (adenine, cytosine, guanine, and thymine) that comprise DNA, but sufficiently different enough to block viral DNA synthesis. There are also non-nucleoside reverse transcriptase inhibitors that can bind to reverse transcriptase and, thus, block the production of viral DNA. Reverse transcriptase inhibitors have been remarkably successful in preventing the spread of HIV from an infected mother to her newborn: if a pregnant woman treated with AZT (a nucleoside analog) delivers her child by caesarian, the chances of the baby being infected can be reduced to one percent.

Protease inhibitors, another major class of drugs, act later in the life cycle of the virus by inhibiting the protease enzyme. These drugs interfere with the cleavage of the viral polypeptide into functional viral enzymes.

The evolution of HIV variants that are resistant to the more commonly used medications has become a major problem. In one study as many as thirty percent of HIV patients harbored resistant viruses. The virus mutates rapidly, and variants that are able to survive in the presence of drug — particularly when circulating levels of the drug are lower — rapidly take over the population. Patient adherence to drug regimens is critical to reducing the emergence of resistant viruses; even the timing of medication can be important. Unfortunately, given the side effects of current treatments, adherence is difficult. Protease inhibitors can cause nausea and diarrhea, and some of the nucleoside reverse transcriptase inhibitors can cause red or white blood cell levels to drop. Painful nerve damage and inflammation of the pancreas can also result.

HAART

Beginning in the mid-1990s, an increasing number of HIV-infected individuals began a drug regime called highly active antiretroviral therapy (**HAART**), a combination of three or more anti-HIV drugs taken at the same time. The simultaneous intake of multiple drugs, each targeting different aspects of the viral life cycle, circumvents the ability of the virus to mutate and become resistant to the drugs. Combined therapies, often called "cocktails," can knock virus back to

undetectable levels and improve patient health significantly. With the advent of HAART, deaths from HIV began to decline in the U.S. in 1997. Unfortunately, HAART has several long-term side effects including kidney, liver, and pancreatic problems; and changes in fat metabolism, which result in elevated cholesterol and triglyceride levels and an increased risk for strokes and heart attacks. In addition, some viruses have evolved resistance to HAART. Given these side effects, some physicians recommend that HAART be delayed until HIV-positive patients are exhibiting clear signs of AIDS. Still, HAART is often recommended in the first few weeks after exposure to bring the initial viral load down.

The treatments described above are directed at the reduction of free virus: they work only against viruses that are actively produced. Because of the latent nature of the virus they are not cures. In addition, treatments are prolonged and may be necessary for a patient's entire life. A patient who stops treatment will typically have an increase in viral numbers.

Also under investigation are treatments that take advantage of our understanding of the process of viral infection. "Fusion" or "entry inhibitors" block the proteins involved in viral uptake, such as CCR5. Integrase inhibitors affect the enzyme necessary for the integration of viral DNA into host DNA. Both have shown promise.

Treatments Based on Understanding the Immune System

Development of novel treatments for HIV also depends on an understanding of the choreography of chemical signals that regulate immune function. Because cellular immunity is key to clearing viral infections, increasing the T cell response is critical to clearing HIV. Interleukin 2 (IL-2) is a cytokine produced by T_H cells that promotes the growth of other T cells. Recombinant IL-2, which has the same activity as the native protein, has been shown to increase CD4 cell numbers in individuals in the early stages of HIV infection. Viral numbers, though, do not seem to go down with this treatment alone. However, IL-2 administered with HAART resulted in more individuals with undetectable viral loads when compared to treatment with HAART alone. One frustration with HIV treatments is the inability to affect cells that harbor provirus. IL-2 administered intermittently to patients with more advanced HIV could work to stimulate viral production and stimulate HIV specific immune responses. Such strategies are under investigation.

Other treatments under consideration target virally infected cells. Some CD8 cells seem to secrete soluble factors that suppress HIV replication. Understanding how these factors work may help define new treatments.

The Challenges of Vaccine Development

Scientists have taken a number of approaches to the development of a vaccine for HIV, but the nature of the virus presents significant challenges. HIV infects only humans and chimpanzees. Evaluating vaccine effectiveness in the chimpanzee model is problematic for several reasons. Chimpanzees are scarce, expensive, and do not show signs of disease when infected. There are also ethical concerns raised

because chimpanzees are our closest evolutionary relatives. An alternative is the development of a monkey model using simian immunodeficiency virus (SIV) that has been genetically engineered to express HIV components. The downside to this approach is the difficulty of predicting what will happen when a vaccine that was developed using monkey models is administered to humans.

The route of transmission of HIV also presents a challenge for vaccine producers. Typically, an individual is exposed to the virus at a mucosal surface where a particular type of antibody molecule, IgA, mediates immunity. The ideal vaccine should stimulate production of this type of antibody, not just the type found in the circulation (IgG). But even if a vaccine stimulates the production of the appropriate type of antibody, an increasing number of investigators are convinced that it may not be enough. Circulating antibodies cannot clear a latent virus, and infected cells seem to persist in the body for long periods. So it may be necessary to stimulate cellular as well as humoral immunity. Another challenge to vaccine production is the variety of viral subtypes. Because distinct HIV subtypes are more prevalent in certain locations, some scientists have asked whether HIV vaccines need to be developed specifically for certain geographical regions. Alternately, immune stimulation must be accomplished using an antigen, or antigens, common to all subtypes.

Another major impediment to vaccine development is HIV's rapid mutation rate and the presence of multiple viral variants within a given individual. Traditional vaccines, such as those for childhood illnesses, consist of live attenuated (weakened) pathogens, dead pathogens, or parts of organisms. Attenuated HIV vaccines are not likely to be pursued because of the risk of infection — whole, killed HIV is a safer alternative. But, given the rapid mutation rate of the virus, many believe that a variant of the virus unaffected by the immune response would evolve quickly.

Vaccines based on pieces of HIV are safer and easier to prepare. Many efforts have been directed to the production of recombinant HIV proteins that can serve as vaccines. For example, vaccines consisting of the gp120 surface protein, which is needed for virus to adhere to cells, could elicit an immune response, inhibiting viral adherence. Unfortunately, gp120 vaccines may not be successful: the site on gp120 that binds CD4 and CCR5 is apparently buried in a molecular pocket, which is not blocked by antibody.

The AIDS epidemic has spurred additional vaccine production strategies that use genetic engineering techniques. Many scientists are examining strategies for generating cellular and humoral immunity; for example, live non-pathogenic bacteria or viruses can be engineered to express HIV antigens. Researchers at Merck Corporation have inserted the *gag* gene, which encodes a viral core protein, into modified adenovirus. They hope that as cell-mediated immunity is mounted against adenovirus, the response will also target HIV-infected cells. The protein encoded by *gag* is among those found unchanged in most HIV variants; therefore, researchers hope that the vaccine could circumvent the genetic variability problem.

The *gag* gene is also the basis of one of several DNA-based vaccines under investigation. Such vaccines contain "naked" DNA (not associated with chromosomes or other structures), which is injected

directly into muscle tissue. The expectation is that some of the DNA will be taken up and expressed by human cells. The immune response directed against these cells is hoped to carry over to HIV-infected cells. Some investigators are combining strategies; for example, Harriet Robinson and her colleagues at Emory University are trying DNA priming, followed by a booster of recombinant pox virus.

Clinical Trials

More than two dozen experimental HIV vaccines are being studied worldwide. For a given vaccine to be proven safe and effective it must pass through three stages of human testing. Phase I addresses safety and dosage, and involves the administration of the vaccine to dozens of people. Phase II examines efficacy, the ability of the vaccine to elicit an immune response, and involves hundreds of people. Phase III involves thousands of people who are followed for a long periods to establish that the vaccine is indeed protective.

At the outset of the AIDS epidemic some scientists anticipated the availability of a vaccine in two or three years. More than twenty years into the epidemic, a vaccine is still down the road and few believe it will be available soon. The idea of distributing a less-than-perfect vaccine is controversial. Some believe protecting only a certain percentage of the population could limit the spread of the disease. Others believe an imperfect vaccine could provide a false sense of security such that individuals might increase risky behaviors.

Social Obstacles to Controlling HIV

Researchers have worked diligently and gained an unprecedented knowledge of the biology of HIV and its interaction with the immune system; yet, the AIDS pandemic will continue for years to come. Obstacles to AIDS prevention and control lie not only in the nature of the HIV virus but the very nature of human societies worldwide. Poverty and discrimination exclude those most in need from information and treatment. The control of HIV lies not only in biology but also in the social realm of basic human rights.

AIDS is having the greatest impact in countries ridden with poverty, where public health infrastructures are already strained by drug-resistant malaria, tuberculosis, yellow fever, Rift Valley fever, and other infectious diseases. (See the *Emerging Infectious Diseases* unit.) Further, the presence of HIV amplifies epidemics of such pathogens. AIDS is the leading cause of death in Africa. In several African countries, more than twenty percent of the 15–49-year-old population is infected with HIV; in Botswana more than thirty percent of that age group is infected. Poverty excludes millions from treatment. Of the roughly 28 million people infected with HIV in sub-Saharan Africa, only 36,000 received drugs in 2002. In response to such statistics drug companies have reduced the cost of treatment to as little as $300–$400 per person in developing countries (treating one person costs at least $10,0000 or more annually in the U.S.) — but even that is too expensive. In 2001 the United Nations launched the Global Fund to Fight HIV, Tuberculosis and Malaria. At the time Kofi Annan, U.N. secretary general, said it would take $7 billion to $10 billion each year to fight HIV/AIDS. As of 2002 the fund, supported mainly by donor nations and philanthropists, had raised only $2 billion.

Poverty is just one obstacle to controlling HIV. Discrimination against particular groups has hindered education, diagnosis, and treatment. The lack of women's rights in some countries has thwarted educational efforts and contributed to the spread of the disease; so has prevailing customs regarding multiple sex partners. Access to basic education, information about HIV transmission, and the power to say no to unwanted sexual advances are as important as access to drugs. Funding for teacher training, education, and prevention materials has been inadequate.

Governments in many countries have been hesitant to implement strong and coordinated AIDS prevention programs. Needle exchange programs for drug users, for example, have been shown in numerous studies to reduce the risk of HIV. Yet, in countries around the world, such programs remain politically unpopular. Condoms protect against transmission of the virus, but promotions of condom use are discouraged by many religious groups and governments. Children around the world are denied access to sex education, mostly for ideological reasons.

By depleting the workforce, AIDS is destabilizing the economies of countries already grappling with poverty and political instability. As people in their twenties and thirties die, countries lose their workers, their teachers, and the parents of their children. Men who have gone to urban areas to work contract HIV, and then return home to give the disease to their wives. Much of the toll of AIDS in Africa is on the women and children, who are critical to maintaining the continent's agricultural economy. In many sub-Saharan countries women are considered their husband's property and have little access to independent income. As men are lost to AIDS their widows become dependent on others or turn to one of the few survival strategies, prostitution. So viral dissemination is amplified and, at the same time, urban and rural economies decline. By the year 2011 there will be 40 million AIDS orphans in Africa. In some countries the illness and death of women in the childbearing years will result in a greatly reduced number of births in the next decades.

In industrialized societies, those touched by AIDS have had considerable impact in fighting the spread of infection. Patients themselves have become active channels for the distribution of information and participate in policy-making and lobbying for funding. But HIV remains latent in those whose voices are repressed. In sub-Saharan Africa, for example, stigma still surrounds people infected with HIV, and silence impedes progress in controlling the epidemic.

HIV is difficult to control because it exploits the immune system designed to stop it and other infections. Researchers are continuing to explore strategies to foil the virus, but treatments and vaccines are just two components to thwarting the epidemic. Policies that ensure accessibility to medications and information are critical. Education is key. AIDS forces us to talk about things we would rather leave unsaid.

References

1. Coffin, J. M. 1999. Molecular biology of HIV. In *The Evolution of HIV*, ed. K. A. Crandall, 3–40. Baltimore: Johns Hopkins University Press.

2. Amborzia, J. and J. A. Levy. 1998. Epidemiology, natural history and Pathogenesis of HIV Infection. In *Sexually Transmitted Diseases,* 3d ed, ed. K.K. Holmes, P.F. Sparling, P.A. Mardh, S.M. Lemon, W.E. Stamm, P. Piot, and J.N. Wasserheit, 251–58. New York: McGraw-Hill.

Further Reading

Book

Garrett, L. 1994. The coming plague — Newly emerging diseases in a world out of balance. New York: Penguin Books.

A detailed book that provides insights into emerging infection through the stories of those intimately involved in a plethora of outbreaks worldwide.

Articles

Amborzia, J. and J. A. Levy. 1998. "Epidemiology, natural history and pathogenesis of HIV infection." Pp 251–58 in *Sexually Transmitted Diseases* (Third Edition), ed. K.K. Holmes, P.F. Sparling, P.A. Mardh, S.M. Lemon, W.E. Stamm, P. Piot, and J.N. Wasserheit. New York: McGraw-Hill.

A review of the transmission and clinical manifestations of HIV infection.

Cimmons, M. 2002. New prospects on the HIV vaccine scene. *ASM News* 68(1):19–22.

An update from the American Society of Microbiology.

Cohen, J. 2002. Confronting the limits of success. *Science* 296 (5577): 2320.

Discusses the successes and challenges regarding HAART (highly active antiretroviral therapy).

Cohen, J. 2002. The high cost of poverty. *Science* 296:2324.

Puts AIDS in terms of dollars and cents.

Ezzel, C. 2002. Hope in a vial. *Scientific American*, June, 38.

An overview of the challenges developing HIV vaccines.

Piot, P. 1998 The science of AIDS: A tale of two worlds. *Science* 280:1844–45.

One of the essays on science and society presented in Science. The essays are directed to the educated lay reader, and can serve as the basis of discussion and debate in the classroom. This essay introduces the impact that policymakers and community members have on the progression of emerging diseases such as AIDS.

Glossary

Antigen. A substance, often a protein or large polysaccharide, which is perceived as foreign by the body and stimulates an immune response. Components of microbes such as cell walls, flagella, toxins, and the coats of viruses can serve as antigens.

CD4. A protein on the surface of certain leukocytes. Helper T cells are among the cell types that possess CD4. CD4 is involved in the binding of HIV to its host cells.

Cellular immunity. The arm of the immune system directed toward antigens associated with cells, such as those expressed by virally infected cells or cancers. The T cells of cellular immunity respond specifically to such antigens.

Chemokine. A chemical signal that attracts white blood cells to infected parts of the body.

Chemokine receptor. A protein associated with the membranes of white blood cells that chemokines can attach to.

Cytokine. A molecular signal that modulates immune response.

dsDNA. Double-stranded DNA. A DNA molecule in which two chains (backbones of alternating sugars and phosphates) are linked together by hydrogen bonding between complementary bases.

gp120. Glycoproteins with a molecular weight of 120,000 daltons, which are part of the peg-like structures protruding from the surface of HIV.

HAART (highly active antiretroviral therapy)**.** A combination of three or more anti-HIV drugs used in the treatment of HIV.

Humoral immunity. The arm of the immune system directed toward circulating antigens such as bacteria, toxins, and viruses that have not entered cells. Antibodies secreted by plasma cells mediate humoral immunity.

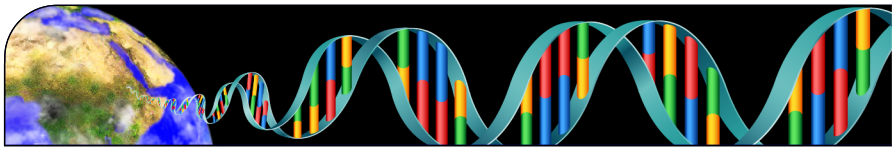
Integrase. An HIV enzyme that facilitates the integration of viral DNA into the host cell's chromosome.

Opsonin. A substance that, when bound to antigen, amplifies the normal phagocytic process.

Protease. An enzyme that facilitates the cleavage of proteins. In HIV, an enzyme that allows for the processing of newly translated polypeptides into the proteins that will be assembled into viral particles.

Provirus. Viral DNA that is incorporated into a host cell's chromosome.

Reverse transcriptase. An enzyme derived from a retrovirus, which uses single-stranded RNA as a template for the production of double-stranded DNA.



REDISCOVERING BIOLOGY

Molecular to Global Perspectives

Genes and Development

"Animals that look nothing like each other develop by using much the same basic 'toolkit' of molecules and often in much the same ways." M. Palopoli and N. Patel[1]

Development poses some of the central questions of biology: How does a single cell become a complex multicellular organism like us? What role do our genes play in the processes of development? From the early decades of the twentieth century, geneticists knew about mutants that altered phenotypes because of the actions of various genes during development. In numerous cases biologists knew where on the chromosome the mutant gene was located and how the mutant allele was transmitted from parent to offspring. Nevertheless, the actual role the genes play in development remained a "black box" mystery until around 1980.

Starting in the late 1970s geneticists figured out the details involved in the genetic control of development in model systems such as the fruitfly *Drosophila melanogaster*. They found that many of these developmental genes shared similar features. During the 1980s and 1990s geneticists made an even more surprising discovery: the same principles, and often the same genes, involved in development in model organisms (such as fruit flies and zebrafish) are also involved in controlling development in most other animals, including humans.

Differentiation and Genetic Cascades

Development of a complex multicellular organism is more than just growth — we certainly do not look like gigantic fertilized eggs. Starting from a single cell, numerous specialized cell types emerge that differ in many ways: size, shape, longevity, biochemistry, and so on. What can account for this great diversity among cell types? What processes underlie this differentiation of a single cell into all the cell types of an adult individual?

Is differentiation due to the loss of certain genes in some cell types? While there are some exceptional cases (for example, mature red blood cells lack nuclei), development, as a rule, is not due to particular cell types having different genes. With only a few exceptions, all the cells in your body contain the same DNA. Discoveries of adult stem cells show that some adult cells retain the potential to produce many, if not all, of the cell types in the organism. These cells can reverse the process of differentiation, reaching a state where their descendants can redifferentiate into all of the cell types.

If cells of an individual are genetically alike, how does differentiation occur? Recall that proteins, not DNA, carry out most cellular functions. (See the *Proteins and Proteomics* unit.) DNA serves a blueprint from which RNA is transcribed. Proteins come from the amino acid chains that are translated from the RNA. The levels of transcription and translation of a gene determine how much of that gene's protein will be present in the cell. Gene expression, which encompasses transcription and translation, is the general term to describe the processes in which DNA produces RNA and proteins. It can also include other factors, such as the rate at which RNA is degraded before it can be translated. Differential gene expression will result in varying concentrations and kinds of proteins in cells, causing them to look and function differently. This differential transcription and translation of genes ultimately allows for cellular differentiation. Thus, development is a program that regulates gene expression at the appropriate locations and times.

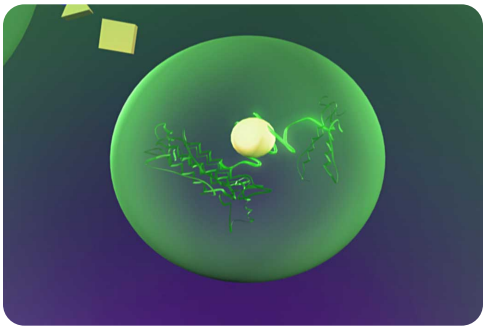
How is it that, for a given cell type, certain subsets of genes are expressed and other genes are not expressed? As we will see later, the protein product that results from the expression of one gene can influence the expression of several other genes. In turn, the altered expression patterns of these genes can then influence the expression of an even larger number of genes. By this process, called a cascade, a change in one or a few genes can alter the expression patterns of numerous genes.

The Details of Gene Expression

What regulates gene expression? The general principles of eukaryotic gene regulation are now well known. Much regulation occurs during transcription as RNA is synthesized from the DNA template. This process is mediated by interactions between proteins and DNA and, sometimes, interactions between different proteins. Proteins called **transcription factors** bind to DNA sequences, known collectively as regulatory elements, located near the coding region of the gene in question (**Fig. 1**). When proteins bind to the **regulatory elements**, it alters the transcriptional machinery and, thus, the level of transcription can change. In some cases the binding of transcription factors to the regulatory elements causes transcription to increase (up-regulation); in other cases it causes transcription to decrease (down-regulation).

The invention of **microarray chips** in the late 1990s enabled researchers to observe the expression patterns of thousands of genes at the same time. (See the *Genomics* unit.) Using these chips, researchers can compare the genomic expression patterns of different cell types (such as a neuron versus a liver cell), as well as examine the changes in these patterns that occur as an embryo develops. With the microarray assays, biologists found many previously undiscovered genes that play a role in development. By examining groups of genes that have correlated changes in their expression patterns, biologists have inferred groups of genes that may interact in **developmental pathways**. They then use other methods to determine whether the hypothetical pathways actually exist.

Figure 1. The yellow ball represents a transcription factor binding to DNA in the nucleus to affect transcription and translation of new proteins.



Establishing the Gradient and Coordinate Genes

Development is a process where the products of some genes turn other genes on or off.

But how does the process start? Even before fertilization, development is occurring. We normally think of an egg as a storehouse of energy supply and nutrients that the embryo will use as it develops. While this is true, the egg also supplies information to establish a molecular coordinate system. This coordinate system provides a way to tell "which end is up"; in other words the location of the embryo's head is determined even before the egg is fertilized.

Coordinate genes are named because they establish the primary coordinate system for what will become the embryo. One important example of a coordinate gene is bicoid, which is involved in establishing the anterior-posterior polarity in *Drosophila*. How does bicoid do this? To understand this process we need to first discuss how bicoid gets to the anterior part of the egg. Nurse cells surround the anterior region of the egg in *Drosophila* and other flies. Cytoplasmic bridges allow various substances — in this case mRNA from bicoid — to be transported from the nurse cells into the egg. The bicoid mRNA is then trapped by proteins produced by other genes. The result is a concentration gradient of bicoid mRNA: the anterior end has the highest concentration and the posterior end lacks it (**Fig. 2**). Translation of bicoid is inhibited until after fertilization, leading to a bicoid protein concentration gradient.

DROSOPHILA EMBRYO WITH BICOID PROTEIN EXPRESSED. Courtesy of Nipam Patel, PhD.

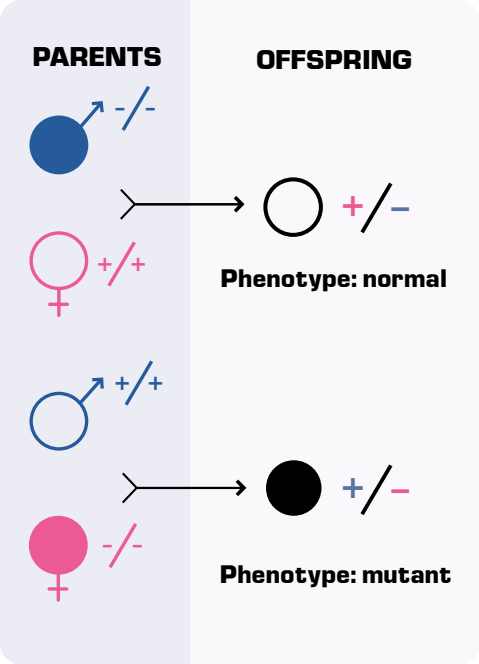
Figure 2. This is a 2-hour-old *Drosophila* embryo that shows the expression of the bicoid protein. The bicoid protein forms a gradient with the highest expression at the anterior end (left side in this photo) of the embryo.

In addition to bicoid, other coordinate genes help establish an anterior-posterior polarity. Still other coordinate genes allow the establishment of a dorsal-ventral gradient.

These coordinate genes, like bicoid, are sometimes called **maternal effect** genes. Maternal effect occurs when the phenotype of the individual is dependent on its mother's genotype, not its own. In cases of maternal effect, the transmission pattern of the alleles is the same as in standard Mendelian genetics but the action of the gene occurs a generation later. For example, consider a maternal effect gene where the mutant allele (m) is recessive to the wild-type allele. In the cross of homozygous, wild-type females to homozygous, mutant males, all the

F1 offspring are heterozygotes and appear normal. In the reciprocal cross, all of the F1 offspring are heterozygotes but have the mutant phenotype (**Fig. 3**). Although the F1 offspring are genotypically identical in the reciprocal crosses, they are phenotypically different. This is because phenotype is due to the action of the mother's genotype. Maternal effect is not the same thing as maternal inheritance — such as in mitochondria, where the genetic material is transmitted only across maternal lines.

Figure 3. Reciprocal F1 crosses involving maternal effect genes can produce different phenotypes.



Responses to the Concentration Gradient

Coordinate genes such as bicoid lay down the grand plan, so to speak, upon which the genes downstream will act. The pattern of the developing embryo arises as these downstream genes are activated or repressed.

Like many of the other coordinate genes, bicoid encodes a transcription factor; thus, there is a concentration gradient of a transcription factor. The next genes in this developmental cascade, the "gap genes," possess binding sites for this transcription factor. Gap genes are so named because mutations in these genes can produce larvae with "gaps" (missing several segments). These genes differ in how many bicoid binding sites they have and, thus, vary in their sensitivity to this transcription factor. Some gap genes will become active at low concentrations of bicoid, while the activation of others will require higher concentrations. Due to the concentration gradient, different regions of the developing embryo will activate different gap genes.

Unlike the coordinate genes, the gap genes are not maternal effect genes. The activities of the embryo's gap genes (and not those of the mother's genes) determine the phenotype. Gap genes also encode for transcription factors, and these affect the transcription of genes that further refine the patterning of the *Drosophila* embryo (**Fig. 4**).

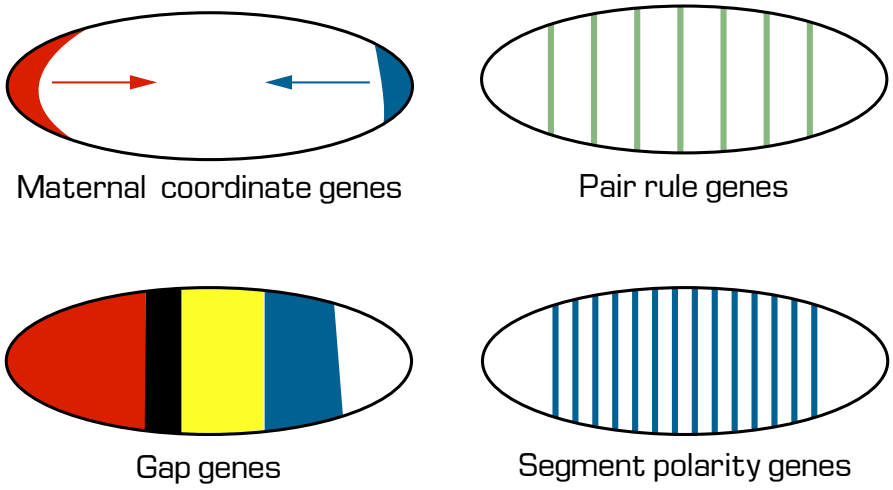


Figure 4. The cascade of developmental genes in segmentation in *Drosophila*. Maternal effect/ coordinate genes set the anterior-posterior axes. The embryo is subdivided into progressively smaller regions by the actions of each class of segmentation genes.

Homeotic Genes

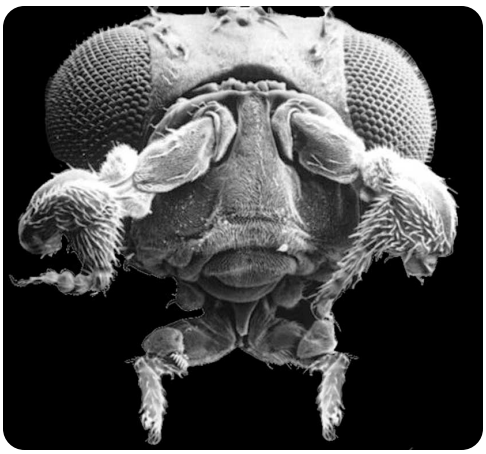
At the end of this cascade is a class of genes that have a long history among *Drosophila* researchers. Decades before Watson and Crick ascertained the structure of DNA, and even more decades before geneticists understood the principles of gene expression, biologists were using *Drosophila melanogaster* as a model system for studying the transmission of genetic traits from parent to offspring. Let's go back to 1915 at Columbia University: In a small laboratory, crowded with thousands of milk bottles containing stocks of the tiny fruitfly *Drosophila melanogaster*, Thomas Hunt Morgan, the father of *Drosophila* genetics, and his students worked. They examined this fruitfly, focusing on ones that looked different in their quest to find and map genes.

One day Calvin Bridges, one of Morgan's graduate students, discovered a most unusual fly. One of the hallmark features of flies is that they have two wings; Diptera, the insect order to which flies belong, means "two wings." The fly Bridges found had one pair of normal wings and one pair of somewhat developed wings. Four wings! Bridges found that this "four wing" phenotype was a genetic mutation that mapped to the third chromosome. After closer inspection, Bridges noted that the third segment of the thorax in these flies looked a good deal like a normal, second segment of the thorax (where wings normally grow). He consequently named the gene associated with this mutant phenotype "bithorax." (Genes in *Drosophila* are traditionally named for their mutant phenotype, not for what they do in normal flies.)

Drosophila geneticists would later find other, similar mutations. One, named ultrabithorax, caused the fly to form two, completely developed pairs of wings. Another seemingly different mutation (antennapedia) caused legs to grow where the fly's antennae should have been (**Fig. 5**). These mutant genes became referred to collectively as **homeotic genes**, named after homeosis. Homeosis, a term coined by William Bateson (a prominent zoologist and one of the early geneticists), refers to "cases in which structures belonging to one body segment were transformed in identity to those belonging to another segment" [2]. Mutants in these genes appeared to change the characteristics of one segment of the fly into those of another segment. Interestingly, all of these genes would map very close together in two clusters on the third chromosome.

Recall the cascade that led to these homeotic genes. The maternal effect coordinate genes laid down the anterior-posterior and dorsal-ventral gradients, which influenced the expression of genes further along the cascade. These genes turned other genes on or off and, as a result, formed the segmented pattern of the *Drosophila* embryo. The homeotic genes, having been turned on or off by genes above in the cascade, are also transcription factors. They influence the expression of numerous other genes and, by doing so, determine the identity of the segment they are in. Certain homeotic genes, such as bithorax, are expressed in what would become the thorax; other genes are expressed only in the head or abdomen. It's interesting to note that genes expressed in similar regions are also located near each other on the chromosome (**Fig. 6**).

Figure 5. A scanning electron microscope image of a *Drosophila* fly with the antennapedia mutation. This mutation causes the fly to grow legs where it should grow antenna.



Thomas Kaufman, PhD, MUTANT DROSOPHILA.
Courtesy of Thomas Kaufman, PhD, University of Indiana.

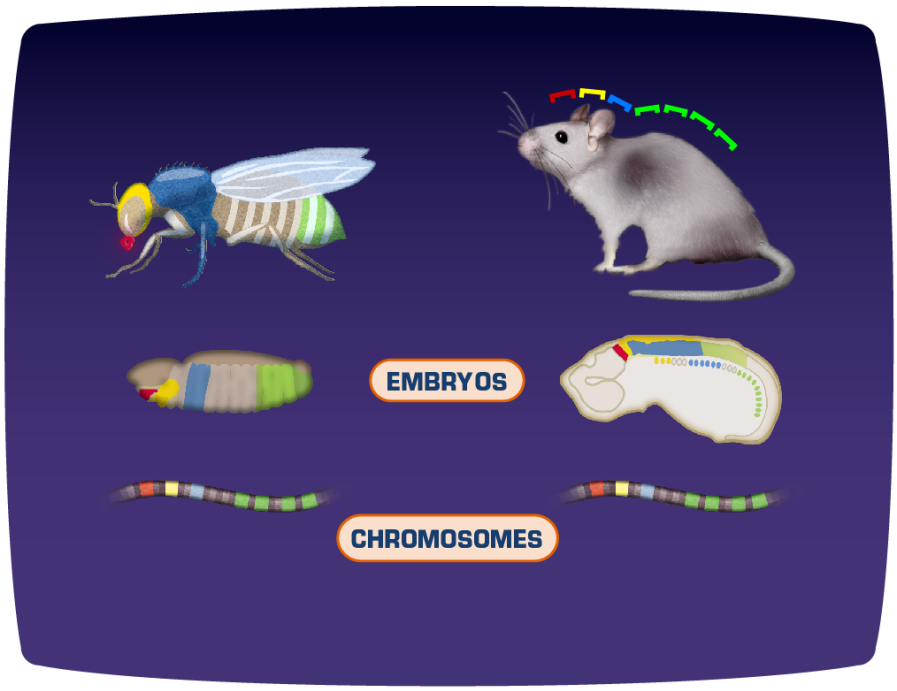


Photo-illustration — Bergmann Graphics

Figure 6. Genes that are expressed at the anterior end of an animal are located at the more anterior region of the chromosome. Likewise, posteriorly expressed genes reside on the posterior end of the chromosome. This is referred to as spatial colinearity.

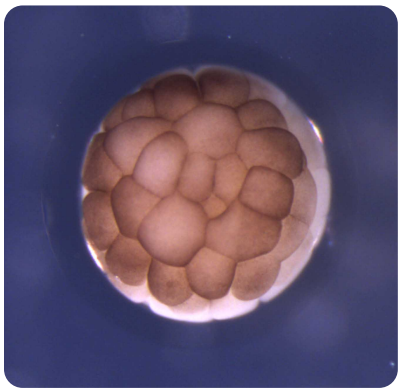
Cell Lineage Mapping and *C. elegans*

Drosophila melanogaster is not the only model organism for developmental genetic studies. Starting in the 1960s geneticists interested in developmental questions turned to a free-living soil nematode, *Caenorhabditis elegans*. This species, usually referred to as just *C. elegans*, has several features that *Drosophila* and most other organisms don't have, which makes it attractive for developmental studies. Because embryos of this nematode are transparent, their cells can be observed easily and without much manipulation. The species also has a low number of cells. In fact, all normal individuals have the same number of cells: 959 somatic cells in the hermaphrodite and 1,031 in the male. Unlike *Drosophila* and mammals, which have extensive cell movement during development, the cells of *C. elegans* do not move very much during development. All of these features made *C. elegans* an ideal organism to study cell lineage history, the ancestral-descendant relationship of cells.

John Sulston and colleagues worked out the entire cell lineage history of C. elegans by 1983. Some cell lineage mutations alter the rate and/or timing of cell division. Others affect differentiation. One remarkable feature of C. elegans development is that seventeen percent of the cells generated during embryogenesis undergo programmed cell death, also called apoptosis. Normal development requires that certain cells die. There are several mutants in which the exact failure of cells to die has been tied to a phenotypic change. Many of the genes involved in programmed cell death in nematodes have counterparts in vertebrates that are also responsible for programmed cell death. Moreover, absence of proper cell death is a key feature of many cancers. (See the Cell Biology and Cancer unit.)

Fate Maps

What Sulston and his colleagues did with tracing the entire cell lineage would be exceedingly difficult for the vast majority of organisms. Most multicellular organisms have far more cells than *C. elegans*. Moreover, most don't have a transparent body or rather sedentary cells during development. Nevertheless, for several different kinds of organisms, researchers have been able to determine the type of tissue that cells in developing embryos will become; **fate maps** are diagrammatic representations of this (**Fig. 7**).



Courtesy of Dr. Anna Philpott, Department of Oncology, Cambridge University.

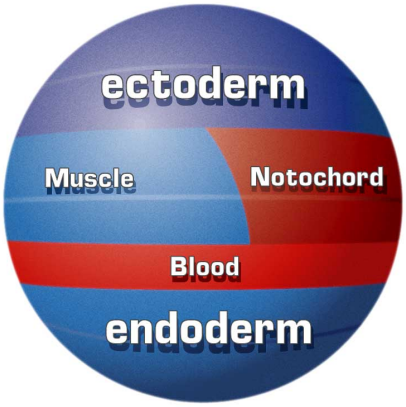


Photo-illustration Bergmann Graphics

Figure 7.
Left: A photograph of an early stage blastula from the *Xenopus laevis* frog. Right: A representation of a fate map.

Scientists have been able to create fate maps for several organisms, such as the sea urchin, since the early decades of the twentieth century. To construct fate maps researchers use various methods, including removing cells from embryos. If the adult that developed from these embryos is missing specific tissues, researchers infer that the removed cells would have become those missing tissues. Researchers can also use a variety of stains to trace cells in the developing embryo.

Cell-Cell Communication and Signal Transduction

Although development begins with a "master plan," initiated by coordinate genes and carried on through a series of genetic cascades, cells also communicate with one another to coordinate development. In addition, cell-cell communication is essential throughout the life of the organism. Indeed, many cancers are due in part to failures of normal cell-cell communication. (See the *Cell Biology and Cancer* unit.)

There are some similarities between the way cells communicate and the way individual organisms communicate: in both cases there are signalers and receivers. Cell-cell communication, like many forms of communication between organisms, involves the transfer of information by using molecules between signalers and receivers. The signaling cell sends out molecules called **ligands**; these can be proteins or small molecules such as vitamin D. Ligands attach to proteins embedded in the membrane of the receiver cell; these proteins are sometimes called receptor proteins.

Once the receptor protein receives the message (the ligand), the nucleus still needs to receive the information because that's where

transcription occurs. How does that happen? Most often, the binding of the ligand causes the receptor protein to change its conformation. This conformational change sets up a series of changes, and sometimes cascades, which eventually lead to changes in transcriptional activity of genes.

One example of a signaling pathway involves the "hedgehog" gene in *Drosophila*. This gene was so named because larvae with the mutant phenotype are covered with hair and look somewhat like a hedgehog. The protein encoded by the hedgehog gene is a ligand and interacts with several receptors. Among other functions, it triggers the early steps in development of postsynaptic neurons. It is also involved in the differentiation of the photoreceptor cells of the eye.

Conservation of the Homeobox

In the early 1980s *Drosophila* geneticists started sequencing the DNA from the homeotic genes. Much to their surprise they found that all the homeotic genes contained a 180-basepair region. This region, named the **homeobox** after the genes in which it was first found, encodes for a sixty-amino-acid sequence that is very well conserved among the homeotic genes. Homeobox refers to the sequence of DNA; the amino acid sequence it encodes is called a homeodomain. Sequences at the homeobox usually differ by ten percent or less between pairs of homeotic genes in Drosophila. Homeoboxes are not restricted to homeotic genes and have been found in several other classes of developmentally important genes. The amino acids encoded by the homeobox region contain a **motif** called a helix-turn-loop, which is associated with binding to DNA sequences. Thus, a gene with a homeobox would be a prime candidate for a gene that encodes a transcription factor.

More surprising than the discovery of homeoboxes themselves was their ubiquity. Soon after homeoboxes were found in *Drosophila*, William McGinnis and his colleagues went on a "fishing" expedition looking for homeotic genes. They looked in a variety of organisms using a method called "zoo blotting," a modified type of **Southern blot**. (See the *Genetically Modified Organisms* unit.) The process consisted of using gel electrophoresis to separate the DNA by size from each species they were interested in. The DNA was then heated to separate it into **single-stranded** DNA (ssDNA). Next the ssDNA was blotted and trapped on nitrocellulose filter paper. The researchers then added single-stranded homeobox DNA, which had been labeled with a radioactive isotope, to the filter paper. If the ssDNA on the blot was sufficiently similar to the labeled homeobox ssDNA, the two ssDNAs would hybridize on the filter paper. The filter paper would be radioactive wherever there was hybridization. To their surprise, McGinnis' group found homeobox sequences everywhere — in insects, crustaceans, vertebrates (including humans and mice), echinoderms, and mollusks. Almost all multicellular animals had genes with homeoboxes. Moreover, they all expressed these genes during development, often in very similar ways.

Most invertebrates have a single cluster of homeotic genes. In *Drosophila* that cluster is broken in two. Vertebrates have four copies of the cluster, strongly suggesting that the cluster had been duplicated twice in vertebrates.

Conservation of the "Control Switch" Gene for Eyes

Phylogenetic analysis has shown that eyes have independently evolved dozens of times in the history of life. (See the *Evolution and Phylogenetics* unit.) For example, there are striking differences between the eyes of insects and those of vertebrates. Vertebrates have a camera eye, consisting of a light-sensitive retina, a lens, and a series of muscles used for adjusting focus. In contrast, insects have compound eyes, consisting of numerous light-sensitive ommatidia.

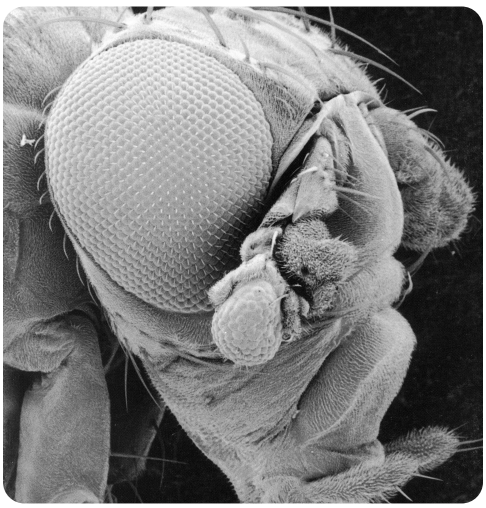
Biologists have learned about the genetics of the visual system in insects by studying mutations that affect eyes in *Drosophila*. Mutants of the eyeless gene in *Drosophila* have reduced eye size, with the extent of the reduction depending on the allele. The eyeless gene is normally expressed only in the tissues that become the eyes. Recall that genes in *Drosophila* are named for the phenotypes of their mutations and not their normal function. What is remarkable about eyeless is that its expression can induce eyes to grow where they ordinarily don't. Members of Walter Gehring's lab in Switzerland created **transgenic** flies, which could express the eyeless gene in various places in the developing fly. By expressing eyeless where it is normally not present (**ectopic expression**), they were able to produce flies with eyes on their antennae, legs, wings, and various other places. So, eyeless looks like a control switch gene for making eyes (**Fig. 8**).

These same researchers also used databases to search for homologous genes of eyeless in mammals. (See the *Genomics* unit.) They found that the eyeless gene in *Drosophila* was strikingly similar (more than ninety percent sequence identity) to the Pax-6 gene in mammals. This Pax-6 gene is also called Smalleyes in mice (where mutants have small eyes) and Aniridia in humans (where mutants lead to deficient development of iris).

Now here's the really fascinating part! Gehring's lab did the same ectopic expression experiment but with the mammalian homologue of eyeless. They produced flies with eyes on their antennae, legs, wings, and various other places. The eyes produced were the compound eyes of flies but the machinery for making these eyes could be turned on by mammalian eyeless protein. Despite the independent evolution of eye structure and over 550 million years of independent evolution, the "control switch" for eye development has been conserved.

There are differences between the role eyeless plays in flies and mammals. Unlike in *Drosophila*, where eyeless is not required for viability, homozygotes for the deletion of eyeless are inviable in mammals. Furthermore, this gene is expressed in regions of the mammalian forebrain. This is strong evidence that eyeless has functions in addition to eye development.

Figure 8. The head of a fruit fly, *Drosophila melanogaster*, viewed by scanning electron microscope (380x magnification). Targeted expression of the eyeless gene induced the formation of the eye facets on the antenna (to the lower-right of the eye), which are very similar to the facets of the normal eye. This dentifies eyeless as the master control gene of eye morphogenesis.



Andreas Hefti and Georg Halder, HEAD OF A FRUIT FLY (1995). Courtesy of *Science* magazine, cover, 24 March 1995.

Sonic Hedgehog

Researchers discovered that vertebrates have a homologue to the *Drosophila* hedgehog gene. They named the vertebrate homologue "Sonic Hedgehog" after the video game character Sonic the Hedgehog. This gene, which encodes a ligand, has diverse functions, including limb development, patterning of the neural tubes (and hence the brain), and differentiation of regions in the gut. How does it

work? Cells of the developing notochord send out Sonic Hedgehog signals to the spinal cord. These cells respond to the signal and then differentiate into the ventral part of the spinal cord, which makes the motor neurons that permit muscular activity. Across mammals this gene is highly conserved; the mouse and human Sonic Hedgehog proteins are ninety-two percent identical at the amino acid level.

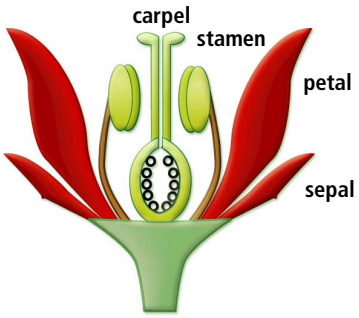
A Brief Look at Plant Development

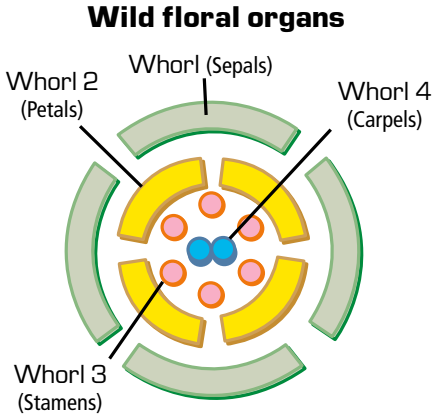
Despite evolving multicellularity independently, plants and animals share some common features in their respective development. These shared features include homeotic mutations and the use of transcription factors. Research in plant development also started with model organisms — in this case, the mustard grass *Arabidopsis thaliana*. In *Arabidopsis* and other plants, the developing flower comprises four concentric whorls. The outermost whorl (Whorl 1) is fated to become the sepals, the outer floral leaves. It surrounds Whorl 2, which is fated to become the petals, the white inner floral leaves. Whorl 3 is fated to become stamens, which contains the male organs. The innermost whorl (Whorl 4) is fated to become the carpels, which will form the ovary (**Fig. 9**).

There are several homeotic mutations in flowers where different parts replace others. For example, in one class of mutations, sepals develop where petals should and carpals develop where stamens should. These mutations have been identified as defects of a family of genes that all encode a particular class of transcription factor, called the MADS box family. MADS box transcription factors occur in both plants and, to a lesser extent, in animals and contain a conserved fifty-eight amino acid sequence.

Plants and animals differ in one important feature: the maintenance of **totipotent** cells. Cells, like the fertilized egg, which can make all of the cells of the organism, are said to be totipotent. In the process of animal development, the competence of the cells to become different cell types declines. But as cells become more differentiated, they continue to lose competence: In animals, **pluripotent** cells can produce most, but not all, types of cells, while **multipotent** cells can produce only a defined set of mature cells. Plants, however, have an apical meristem located at the tip of every root and stem that remains totipotent. They have other meristems that are also totipotent. Moreover, under the right conditions many differentiated plant cells are able to "de-differentiate to the embryonic state and subsequently redifferentiate to new cell types."[3]

Figure 9. The tissues that will become floral organs are arranged in concentric whorls of a developing flower.





Stem Cells

Certainly some plant cells, like the totipotent meristems, are more versatile than animal cells. Recent discoveries, however, show that the difference in the retention of competence between animals and plant cells is not as great as once thought. During the late 1990s scientists found that adult humans have a reservoir of cells that retain some ability to become other cell types.

Cells derived from fetal tissue have been used to generate so-called embryonic stem cells. In addition to the ethical dilemmas raised by the source of embryonic stem cells, there are practical limitations to the use of these cells for treating and curing diseases and regenerating

tissues. Because the donors of these cells are immunologically different from the recipient, immunosuppression would have to be used as it is in organ transplantation. Because adult stem cells can be derived from the individual patient, concerns about compatibility of the cells would be obviated.

But do adult stem cells have the same ability to differentiate as embryonic stem cells? Recent studies suggest that adult stem cells may be more versatile than had been previously thought. Catherine Verfaillie and her colleagues at the University Stem Cell Institute derived what they call Multipotent Adult Progenitor Cells (MAPC) from the bone marrow of adult mice. These cells appear to be able to differentiate into virtually all cell types of mouse when injected into mouse blastocysts. These MAPCs have also been injected into living adult mice and have differentiated into liver, lung, and intestine tissue.

Coda

The fact that the same principles and many of the same genes direct the development of such different and diverse animals has generated renewed interest and study of how developmental systems evolve. Given the striking similarity of genes used, how do the manifest differences across animals arise and evolve? This question will keep biologists busy for many years to come.

References

1) Palopoli, M. F., and N. H. Patel. 1996. Neo-Darwinian developmental evolution: Can we bridge the gap between pattern and process? *Current Opinion in Genetics and Development* 6:502–8.

2) Raff, R. C. 1996. *The Shape of Life: Genes, Development, and the Evolution of Animal Form, 27*. Chicago: University of Chicago Press.

3) Halder, G., P. Callaerts, and W. J. Gehring. 1995. Induction of ectopic eyes by targeted expression of the eyeless gene of *Drosophila*. *Science* 267:1788–92.

Further Reading

Books

Hartl, D. L., and E. W. Jones. 2001. *Genetics: Analysis of genes and genomes*. 5th ed. Sudbury, MA: Jones and Bartlett.
A college-level textbook on the principles of genetics; includes an overview of the genetic control of development.

Griffiths, A. J. F., J. H. Miller, D. T. Suzuki, R. C. Lewontin, and W. M. Gelbart. 2000. *An introduction to genetic analysis*. 7th ed. New York: W. H. Freeman and Company.
A textbook that provides more in-depth, but somewhat more difficult coverage, of developmental genetics.

Raff, R. C. 1996. *The shape of life: Genes, development, and the evolution of animal form*. Chicago: University of Chicago Press.
A look at the evolution of development.

Glossary

Coordinate genes. Genes that set the coordinate system, the primary anterior-posterior and dorsal-ventral axes, of the early embryo.

Developmental pathway. A sequence of genes that underlie a developmental process.

Differentiation. The process by which cells specialize during development.

Ectopic expression. Expression (transcription and translation) of a gene at a time or place where it is normally not expressed.

Fate map. The diagrammatic representation of the cells in the embryo and the eventual type of tissue they will become in the adult.

Homeobox. A 180-nucleotide section of DNA that codes for a specific class of DNA-binding proteins; first found in the homeotic genes of *Drosophila melanogaster*.

Homeotic genes. Early developmental genes that specify segment identity.

Ligand. A molecule that binds to a protein, usually at a specific binding site.

Maternal effect. The condition where the phenotype is determined not by the individual's own genotype but by its mother's genotype.

Microarray chip. Set of miniaturized biochemical reactions that occur in small spots on a microscope slide, which may be used to test DNA fragments, antibodies, or proteins.

Motif. A short region in a protein sequence, which is conserved in many proteins.

Multipotent. Cells that can produce a defined set of cell types.

Pluripotent. Cells that can produce most, but not all, types of cells of the adult organism.

Programmed cell death. Death of cells that is part of the normal process of development of an organism. (See also apoptosis from Cancer unit,)

Regulatory element. Sequences near the coding regions of genes to which transcription factors can bind, thus influencing transcription.

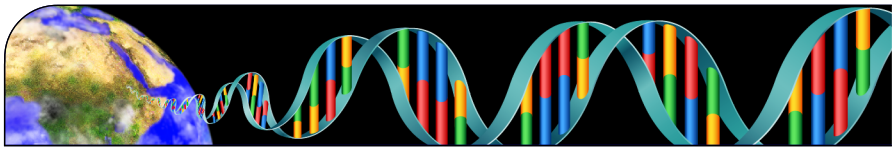
Southern blot. A technique for transferring DNA fragments separated by electrophoresis to a filter paper sheet. The fragments are then probed with a labeled, complementary nucleic acid to help determine their positions.

ssDNA. Single-stranded DNA. A DNA molecule consisting of only one chain of alternating sugars (deoxyribose) and phosphates.

Totipotent. Cells that can replicate to form any part of a complete organism.

Transcription factor. A protein that influences transcription of another gene by binding to DNA.

Transgenic organism. An organism that contains hereditary information from two different species of organisms.



REDISCOVERING BIOLOGY

Molecular to Global Perspectives

Cell Biology and Cancer

"We now understand a lot about cancer. We know that it results from a series of genetic changes having to do with cell division and growth control and genetic instability, mortality, the suicide mechanism in cells; the ability of the cells to migrate; the ability of the cells to attract to them a blood supply. And so that's pretty profound that in a few sentences one can summarize a sophisticated, fundamental understanding of what a cancer is." LELAND HARTWELL

Introduction

A multicellular organism can thrive only when all its cells function in accordance with the rules that govern cell growth and reproduction. Why does a normal cell suddenly become a "rebel," breaking the rules, dividing recklessly, invading other tissues, usurping resources, and in some cases eventually killing the body in which it lives?

To understand how and why cells rebel, we need to understand the normal functions of cell growth and reproduction. From the mid-nineteenth century on, research in cell biology, biochemistry, and molecular biology has provided astonishingly detailed information about the molecules and processes that allow cells to divide, grow, differentiate, and perform their essential functions. This basic knowledge of cell biology has also led to practical discoveries about the mechanisms of cancer. Specific molecules that control the progression of a cell through the cell cycle regulate cell growth. An understanding of normal cell cycle processes and how those processes go awry provides key information about the mechanisms that trigger cancer. Loss of control of the cell cycle is one of the critical steps in the development of cancer.

Although cancer comprises at least 100 different diseases, all cancer cells share one important characteristic: they are abnormal cells in which the processes regulating normal cell division are disrupted. That is, cancer develops from changes that cause normal cells to acquire abnormal functions. These changes are often the result of inherited mutations or are induced by environmental factors such as UV light, X-rays, chemicals, tobacco products, and viruses. All evidence suggests that most cancers are not the result of one single event or factor. Rather, around four to seven events are usually required for a normal cell to evolve through a series of premalignant stages into an invasive cancer. Often many years elapse between the initial event and

the development of cancer. The development of molecular biological techniques may help in the diagnosis of potential cancers in the early stages, long before tumors are visible.

What Is Cancer?

Cancer results from a series of molecular events that fundamentally alter the normal properties of cells. In cancer cells the normal control systems that prevent cell overgrowth and the invasion of other tissues are disabled. These altered cells divide and grow in the presence of signals that normally inhibit cell growth; therefore, they no longer require special signals to induce cell growth and division. As these cells grow they develop new characteristics, including changes in cell structure, decreased cell adhesion, and production of new enzymes. These heritable changes allow the cell and its progeny to divide and grow, even in the presence of normal cells that typically inhibit the growth of nearby cells. Such changes allow the cancer cells to spread and invade other tissues.

The abnormalities in cancer cells usually result from mutations in protein-encoding genes that regulate cell division. Over time more genes become mutated. This is often because the genes that make the proteins that normally repair DNA damage are themselves not functioning normally because they are also mutated. Consequently, mutations begin to increase in the cell, causing further abnormalities in that cell and the daughter cells. Some of these mutated cells die, but other alterations may give the abnormal cell a selective advantage that allows it to multiply much more rapidly than the normal cells. This enhanced growth describes most cancer cells, which have gained functions repressed in the normal, healthy cells. As long as these cells remain in their original location, they are considered benign; if they become invasive, they are considered malignant. Cancer cells in malignant tumors can often metastasize, sending cancer cells to distant sites in the body where new tumors may form.

Genetics of Cancer

Only a small number of the approximately 35,000 genes in the human genome have been associated with cancer. (See the *Genomics* unit.) Alterations in the same gene often are associated with different forms of cancer. These malfunctioning genes can be broadly classified into three groups. The first group, called **proto-oncogenes**, produces protein products that normally enhance cell division or inhibit normal cell death. The mutated forms of these genes are called **oncogenes**. The second group, called **tumor suppressors**, makes proteins that normally prevent cell division or cause cell death. The third group contains DNA repair genes, which help prevent mutations that lead to cancer.

Proto-oncogenes and tumor suppressor genes work much like the accelerator and brakes of a car, respectively. The normal speed of a car can be maintained by controlled use of both the accelerator and the brake. Similarly, controlled cell growth is maintained by regulation of proto-oncogenes, which accelerate growth, and tumor suppressor genes, which slow cell growth. Mutations that produce oncogenes accelerate growth while those that affect tumor suppressors prevent the normal inhibition of growth. In either case, uncontrolled cell growth occurs.

Oncogenes and Signal Transduction

In normal cells, proto-oncogenes code for the proteins that send a signal to the nucleus to stimulate cell division. These signaling proteins act in a series of steps called signal transduction cascade or pathway **(Fig. 1).** (See the *Genetics and Development* unit.) This cascade includes a membrane receptor for the signal molecule, intermediary proteins that carry the signal through the cytoplasm, and **transcription factors** in the nucleus that activate the genes for cell division. In each step of the pathway, one factor or protein activates the next; however, some factors can activate more than one protein in the cell. Oncogenes are altered versions of the proto-oncogenes that code for these signaling molecules. The oncogenes activate the signaling cascade continuously, resulting in an increased production of factors that stimulate growth. For instance, *MYC* is a proto-oncogene that codes for a transcription factor. Mutations in *MYC* convert it into an oncogene associated with seventy percent of cancers. *RAS* is another oncogene that normally functions as an "on-off" switch in the signal cascade. Mutations in *RAS* cause the signaling pathway to remain "on," leading to uncontrolled cell growth. About thirty percent of tumors — including lung, colon, thyroid, and pancreatic carcinomas — have a mutation in *RAS*.

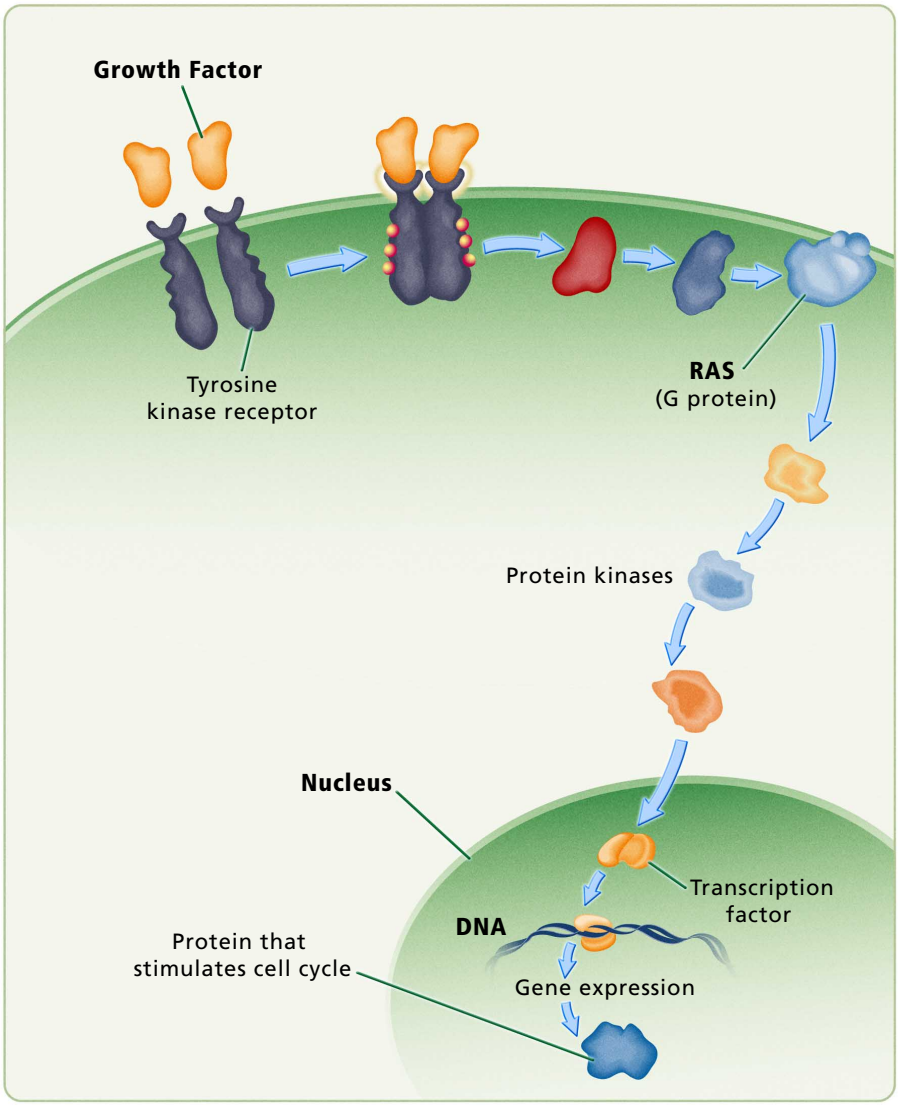


Photo-illustration — Bergmann Graphics

Figure 1. Signal transduction pathway. A signal (in this example, a growth factor) binds to a tyrosine kinase receptor on the outside of the cell. This activates the membrane protein (through the addition of phosphate groups), which in turn activates proteins, such as kinases, in the cytoplasm. Several other proteins may be involved in the cascade, ultimately activating one or more transcription factors. The activated transcription factors enter the nucleus where they stimulate the expression of the genes that are under the control of that factor. This is an example of the *RAS* pathway, which results in cell division.

The conversion of a proto-oncogene to an oncogene may occur by mutation of the proto-oncogene, by rearrangement of genes in the chromosome that moves the proto-oncogene to a new location, or by an increase in the number of copies of the normal proto-oncogene. Sometimes a virus inserts its DNA in or near the proto-oncogene, causing it to become an oncogene. The result of any of these events is an altered form of the gene, which contributes to cancer. Think again of the analogy of the accelerator: mutations that convert proto-oncogenes into oncogenes result in an accelerator stuck to the floor, producing uncontrolled cell growth.

Most oncogenes are dominant mutations; a single copy of this gene is sufficient for expression of the growth trait. This is also a "gain of function" mutation because the cells with the mutant form of the protein have gained a new function not present in cells with the normal gene. If your car had two accelerators and one were stuck to the floor, the car would still go too fast, even if there were a second, perfectly functional accelerator. Similarly, one copy of an oncogene is sufficient to cause alterations in cell growth. The presence of an oncogene in a germ line cell (egg or sperm) results in an inherited predisposition for tumors in the offspring. However, a single oncogene is not usually sufficient to cause cancer, so inheritance of an oncogene does not necessarily result in cancer.

Tumor Suppressor Genes

The proteins made by tumor suppressor genes normally inhibit cell growth, preventing tumor formation. Mutations in these genes result in cells that no longer show normal inhibition of cell growth and division. The products of tumor suppressor genes may act at the cell membrane, in the cytoplasm, or in the nucleus. Mutations in these genes result in a loss of function (that is, the ability to inhibit cell growth) so they are usually recessive. This means that the trait is not expressed unless both copies of the normal gene are mutated. Using the analogy to a car, a mutation in a tumor suppressor gene acts much like a defective brake: if your car had two brakes and only one was defective, you could still stop the car.

How is it that both genes can become mutated? In some cases, the first mutation is already present in a germ line cell (egg or sperm); thus, all the cells in the individual inherit it. Because the mutation is recessive, the trait is not expressed. Later a mutation occurs in the second copy of the gene in a somatic cell. In that cell both copies of the gene are mutated and the cell develops uncontrolled growth. An example of this is hereditary retinoblastoma, a serious cancer of the retina that occurs in early childhood. When one parent carries a mutation in one copy of the *RB* tumor suppressor gene, it is transmitted to offspring with a fifty percent probability. About ninety percent of the offspring who receive the one mutated *RB* gene from a parent also develop a mutation in the second copy of *RB*, usually very early in life. These individuals then develop retinoblastoma. Not all cases of retinoblastoma are hereditary: it can also occur by mutation of both copies of *RB* in the somatic cell of the individual. Because retinoblasts are rapidly dividing cells and there are thousands of them, there is a high incidence of a mutation in the second copy of *RB* in individuals who inherited one mutated copy. This disease afflicts only young children because only individuals younger than about eight years old

have retinoblasts. In adults, however, mutations in *RB* may lead to a predisposition to several other forms of cancer.

Three other cancers associated with defects in tumor suppressor genes include familial adenomatous polyposis of the colon (FPC), which results from mutations to both copies of the *APC* gene; hereditary breast cancer, resulting from mutations to both copies of *BRCA2*; and hereditary breast and ovarian cancer, resulting from mutations to both copies of *BRCA1*. While these examples suggest that heredity is an important factor in cancer, the majority of cancers are sporadic with no indication of a hereditary component. Cancers involving tumor suppressor genes are often hereditary because a parent may provide a germ line mutation in one copy of the gene. This may lead to a higher frequency of loss of both genes in the individual who inherits the mutated copy than in the general population. However, mutations in both copies of a tumor suppressor gene can occur in a somatic cell, so these cancers are not always hereditary. Somatic mutations that lead to loss of function of one or both copies of a tumor suppressor gene may be caused by environmental factors, so even these familial cancers may have an environmental component.

Table 1. Some Genes Associated with Cancer

NAME	FUNCTION	EXAMPLES of Cancer/Diseases	TYPE of Cancer Gene
APC	regulates transcription of target genes	Familial Adenomatous Polyposis	tumor suppressor
BCL2	involved in apoptosis; stimulates angiogenesis	Leukemia; Lymphoma	oncogene
BLM	DNA repair	Bloom Syndrome	DNA repair
BRCA1	may be involved in cell cycle control	Breast, Ovarian, Prostatic, & Colonic Neoplasms	tumor suppressor
BRCA2	DNA repair	Breast & Pancreatic Neoplasms; Leukemia	tumor suppressor
HER2	tyrosine kinase; growth factor receptor	Breast, Ovarian Neoplasms	oncogene
MYC	involved in protein-protein interactions with various cellular factors	Burkitt's Lymphoma	oncogene
p16	cyclin-dependent kinase inhibitor	Leukemia; Melanoma; Multiple Myeloma; Pancreatic Neoplasms	tumor suppressor
p21	cyclin-dependent kinase inhibitor		tumor suppressor
p53	apoptosis; transcription factor	Colorectal Neoplasms; Li-Fraumeni Syndrome	tumor suppressor
RAS	GTP-binding protein; important in signal transduction cascade	Pancreatic, Colorectal, Bladder Breast, Kidney, & Lung Neoplasms; Leukemia; Melanoma	oncogene
RB	regulation of cell cycle	Retinoblastoma	tumor suppressor
SIS	growth factor	Dermatofibrosarcoma; Meningioma; Skin Neoplasms	oncogene
XP	DNA repair	Xeroderma pigmentosum	DNA repair

DNA Repair Genes

A third type of gene associated with cancer is the group involved in DNA repair and maintenance of chromosome structure. Environmental factors, such asionizing radiation, UV light, and chemicals, can damage DNA. Errors in DNA replication can also lead to mutations. Certain gene products repair damage to chromosomes, thereby minimizing mutations in the cell. When a DNA repair gene is mutated its product is no longer made, preventing DNA repair and allowing further mutations to accumulate in the cell. These mutations can increase the

frequency of cancerous changes in a cell. A defect in a DNA repair gene called *XP* (Xeroderma pigmentosum) results in individuals who are very sensitive to UV light and have a thousand-fold increase in the incidence of all types of skin cancer. There are seven *XP* genes, whose products remove DNA damage caused by UV light and other carcinogens. Another example of a disease that is associated with loss of DNA repair is Bloom syndrome, an inherited disorder that leads to increased risk of cancer, lung disease, and diabetes. The mutated gene in Bloom syndrome, *BLM*, is required for maintaining the stable structure of chromosomes. Individuals with Bloom syndrome have a high frequency of chromosome breaks and interchanges, which can result in the activation of oncogenes.

Cell Cycle

Normal cells grow and divide in an orderly fashion, in accordance with the cell cycle. (Mutations in proto-oncogenes or in tumor suppressor genes allow a cancerous cell to grow and divide without the normal controls imposed by the cell cycle.) The major events in the cell cycle are described in **Fig. 2**.

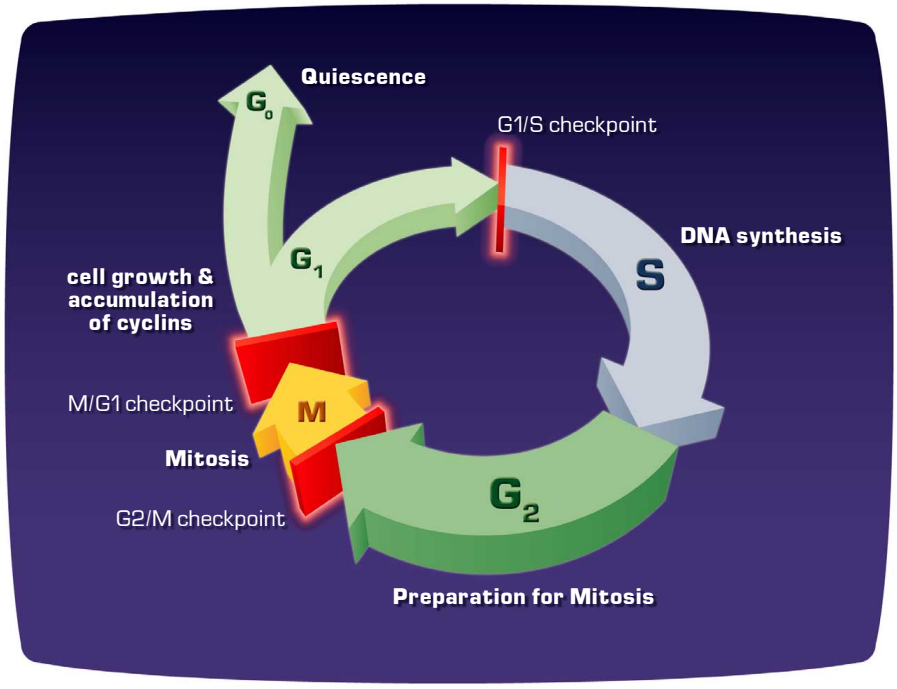


Photo-illustration — Bergmann Graphics

Figure 2. The cell cycle is an ordered process of events that occurs in four stages. During the two gap phases, G1 and G2, the cell is actively metabolizing but not dividing. In S (synthesis) phase, the chromosomes duplicate as a result of DNA replication. During the M (mitosis) phase, the chromosomes separate in the nucleus and the division of the cytoplasm (cytokinesis) occurs. There are checkpoints in the cycle at the end of G1 and G2 that can prevent the cell form entering the S or M phases of the cycle. Cells that are not in the process of dividing are in the G0 stage, which includes most adult cells.

Several proteins control the timing of the events in the cell cycle, which is tightly regulated to ensure that cells divide only when necessary. The loss of this regulation is the hallmark of cancer. Major control switches of the cell cycle are **cyclin-dependent kinases.** Each cyclin-dependent kinase forms a complex with a particular **cyclin**, a protein that binds and activates the cyclin-dependent kinase. The **kinase** part of the complex is an enzyme that adds a phosphate to various proteins required for progression of a cell through the cycle. These added phosphates alter the structure of the protein and can activate or inactivate the protein, depending on its function. There are specific cyclin-dependent kinase/cyclin complexes at the entry points into the G1, S, and M phases of the cell cycle, as well as additional factors that help prepare the cell to enter S phase and M phase.

One important protein in the cell cycle is *p53*, a transcription factor (see the *Genes and Development* unit) that binds to DNA, activating transcription of a protein called *p21*. *P21* blocks the activity of a cyclin-dependent kinase required for progression through G1. This block allows time for the cell to repair the DNA before it is replicated. If the DNA damage is so extensive that it cannot be repaired, *p53* triggers the cell to commit suicide. The most common mutation leading to cancer is in the gene that makes *p53*. Li-Fraumeni syndrome, an inherited predisposition to multiple cancers, results from a germ line (egg or sperm) mutation in *p53*. Other proteins that stop the cell cycle by inhibiting cyclin dependent kinases are *p16* and *RB*. All of these proteins, including *p53*, are tumor suppressors.

Cancer cells do not stop dividing, so what stops a normal cell from dividing? In terms of cell division, normal cells differ from cancer cells in at least four ways.

- Normal cells require external growth factors to divide. When synthesis of these growth factors is inhibited by normal cell regulation, the cells stop dividing. Cancer cells have lost the need for positive growth factors, so they divide whether or not these factors are present. Consequently, they do not behave as part of the tissue — they have become independent cells.
- Normal cells show contact inhibition; that is, they respond to contact with other cells by ceasing cell division. Therefore, cells can divide to fill in a gap, but they stop dividing as soon as there are enough cells to fill the gap. This characteristic is lost in cancer cells, which continue to grow after they touch other cells, causing a large mass of cells to form.
- Normal cells age and die, and are replaced in a controlled and orderly manner by new cells. Apoptosis is the normal, programmed death of cells. Normal cells can divide only about fifty times before they die. This is related to their ability to replicate DNA only a limited number of times. Each time the chromosome replicates, the ends (telomeres) shorten. In growing cells, the enzyme telomerase replaces these lost ends. Adult cells lack telomerase, limiting the number of times the cell can divide. However, telomerase is activated in cancer cells, allowing an unlimited number of cell divisions.
- Normal cells cease to divide and die when there is DNA damage or when cell division is abnormal. Cancer cells continue to divide, even when there is a large amount of damage to DNA or when the cells are abnormal. These progeny cancer cells contain the abnormal DNA; so, as the cancer cells continue to divide they accumulate even more damaged DNA.

What Causes Cancer?

The prevailing model for cancer development is that mutations in genes for tumor suppressors and oncogenes lead to cancer. However, some scientists challenge this view as too simple, arguing that it fails to explain the genetic diversity among cells within a single tumor and does not adequately explain many chromosomal aberrations typical of cancer cells. An alternate model suggests that there are "master genes" controlling cell division. A mutation in a master gene leads to abnormal replication of chromosomes, causing whole sections of chromosomes to be missing or duplicated. This leads to a change in

gene dosage, so cells produce too little or too much of a specific protein. If the chromosomal aberrations affect the amount of one or more proteins controlling the cell cycle, such as growth factors or tumor suppressors, the result may be cancer. There is also strong evidence that the excessive addition of methyl groups to genes involved in the cell cycle, DNA repair, and apoptosis is characteristic of some cancers. There may be multiple mechanisms leading to the development of cancer. This further complicates the difficult task of determining what causes cancer.

Tumor Biology

Cancer cells behave as independent cells, growing without control to form tumors. Tumors grow in a series of steps. The first step is **hyperplasia**, meaning that there are too many cells resulting from uncontrolled cell division. These cells appear normal, but changes have occurred that result in some loss of control of growth. The second step is **dysplasia**, resulting from further growth, accompanied by abnormal changes to the cells. The third step requires additional changes, which result in cells that are even more abnormal and can now spread over a wider area of tissue. These cells begin to lose their original function; such cells are called **anaplastic**. At this stage, because the tumor is still contained within its original location (called *in situ*) and is not invasive, it is not considered malignant — it is *potentially* malignant. The last step occurs when the cells in the tumor metastasize, which means that they can invade surrounding tissue, including the bloodstream, and spread to other locations. This is the most serious type of tumor, but not all tumors progress to this point. Non-invasive tumors are said to be benign.

The type of tumor that forms depends on the type of cell that was initially altered. There are five types of tumors.

- Carcinomas result from altered epithelial cells, which cover the surface of our skin and internal organs. Most cancers are carcinomas.
- Sarcomas result from changes in muscle, bone, fat, or connective tissue.
- Leukemia results from malignant white blood cells.
- Lymphoma is a cancer of the lymphatic system cells that derive from bone marrow.
- Myelomas are cancers of specialized white blood cells that make antibodies.

Angiogenesis

Although tumor cells are no longer dependent on the control mechanisms that govern normal cells, they still require nutrients and oxygen in order to grow. All living tissues are amply supplied with capillary vessels, which bring nutrients and oxygen to every cell. As tumors enlarge, the cells in the center no longer receive nutrients from the normal blood vessels. To provide a blood supply for all the cells in the tumor, it must form new blood vessels to supply the cells in the center with nutrients and oxygen. In a process called **angiogenesis**, tumor cells make growth factors which induce formation of new capillary blood vessels. The cells of the blood vessels that divide to make new capillary vessels are inactive in normal tissue; however,

tumors make angiogenic factors, which activate these blood vessel cells to divide. Without the additional blood supplied by angiogenesis, tumors can grow no larger than about half a millimeter.

Without a blood supply, tumor cells also cannot spread, or metastasize, to new tissues. Tumor cells can cross through the walls of the capillary blood vessel at a rate of about one million cells per day. However, not all cells in a tumor are angiogenic. Both angiogenic and non-angiogenic cells in a tumor cross into blood vessels and spread; however, non-angiogenic cells give rise to dormant tumors when they grow in other locations. In contrast, the angiogenic cells quickly establish themselves in new locations by growing and producing new blood vessels, resulting in rapid growth of the tumor.

How do tumors begin to produce angiogenic factors? An oncogene called *BCL2* has been shown to greatly increase the production of a potent stimulator of angiogenesis. It appears, then, that oncogenes in tumor cells may cause an increased expression of genes that make angiogenic factors. There are at least fifteen angiogenic factors and production of many of these is increased by a variety of oncogenes. Therefore, oncogenes in some tumor cells allow those cells to produce angiogenic factors. The progeny of these tumor cells will also produce angiogenic factors, so the population of angiogenic cells will increase as the size of the tumor increases.

How important is angiogenesis in cancer? Dormant tumors are those that do not have blood vessels; they are generally less than half a millimeter in diameter. Several autopsy studies in which trauma victims were examined for such very small tumors revealed that thirty-nine percent of women aged forty to fifty have very small breast tumors, while forty-six percent of men aged sixty to seventy have very small prostate tumors. Amazingly, ninety-eight percent of people aged fifty to seventy have very small thyroid tumors. However, for those age groups in the general population, the incidence of these particular cancers is only one-tenth of a percent (thyroid) or one percent (breast or prostate cancer). The conclusion is that the incidence of dormant tumors is very high compared to the incidence of cancer. Therefore, angiogenesis is critical for the progression of dormant tumors into cancer.

Viruses and Cancer

Many viruses infect humans but only a few viruses are known to promote human cancer. These include both DNA viruses and retroviruses, a type of RNA virus. (See the *HIV and AIDS* unit.) Viruses associated with cancer include human papillomavirus (genital carcinomas), hepatitis B (liver carcinoma), Epstein-Barr virus (Burkitt's lymphoma and nasopharyngeal carcinoma), human T-cell leukemia virus (T-cell lymphoma); and, probably, a herpes virus called KSHV (Kaposi's sarcoma and some B cell lymphomas). The ability of retroviruses to promote cancer is associated with the presence of oncogenes in these viruses. These oncogenes are very similar to proto-oncogenes in animals. Retroviruses have acquired the proto-oncogene from infected animal cells. An example of this is the normal cellular *c-SIS* proto-oncogene, which makes a cell growth factor. The viral form of this gene is an oncogene called *v-SIS*. Cells infected with the virus that has *v-SIS* overproduce the growth factor, leading to high levels of cell growth and possible tumor cells.

Viruses can also contribute to cancer by inserting their DNA into a chromosome in a host cell. Insertion of the virus DNA directly into a proto-oncogene may mutate the gene into an oncogene, resulting in a tumor cell. Insertion of the virus DNA near a gene in the chromosome that regulates cell growth and division can increase transcription of that gene, also resulting in a tumor cell. Using a different mechanism, human papillomavirus makes proteins that bind to two tumor suppressors, *p53 protein* and *RB protein,* transforming these cells into tumor cells. Remember that these viruses *contribute* to cancer, they do not by themselves cause it. Cancer, as we have seen, requires several events.

Environmental Factors

Several environmental factors affect one's probability of acquiring cancer. These factors are considered carcinogenic agents when there is a consistent correlation between exposure to an agent and the occurrence of a specific type of cancer. Some of these carcinogenic agents include X-rays, UV light, viruses, tobacco products, pollutants, and many other chemicals. X-rays and other sources of radiation, such as radon, are carcinogens because they are potent mutagens. Marie Curie, who discovered radium, paving the way for radiation therapy for cancer, died of cancer herself as a result of radiation exposure in her research. Tobacco smoke contributes to as many as half of all cancer deaths in the U.S., including cancers of the lung, esophagus, bladder, and pancreas. UV light is associated with most skin cancers, including the deadliest form, melanoma. Many industrial chemicals are carcinogenic, including benzene, other organic solvents, and arsenic. Some cancers associated with environmental factors are preventable. Simply understanding the danger of carcinogens and avoiding them can usually minimize an individual's exposure to these agents.

The effect of environmental factors is not independent of cancer genes. Sunlight alters tumor suppressor genes in skin cells; cigarette smoke causes changes in lung cells, making them more sensitive to carcinogenic compounds in smoke. These factors probably act directly or indirectly on the genes that are already known to be involved in cancer. Individual genetic differences also affect the susceptibility of an individual to the carcinogenic affects of environmental agents. About ten percent of the population has an alteration in a gene, causing them to produce excessive amounts of an enzyme that breaks down hydrocarbons present in smoke and various air pollutants. The excess enzyme reacts with these chemicals, turning them into carcinogens. These individuals are about twenty-five times more likely to develop cancer from hydrocarbons in the air than others are.

Detecting and Diagnosing Cancer

The most common techniques for detecting cancer are imaging techniques such as MRI, X-rays (such as mammograms), CT, and ultrasound, which can provide an image of a tumor. Endoscopy allows a physician to insert a lighted instrument to look for tumors in organs such as the stomach, colon, and lungs. Most of these techniques are used to detect visible tumors, which must then be removed by biopsy and examined microscopically by a pathologist. The pathologist looks for abnormalities in the cells in terms of their shape, size, and

structure, especially the nucleus. In addition, the pathologist looks at the borders of the tumor to see whether those cells are normal. Based on examination of the tumor cells, the pathologist determines whether the tumor is benign or malignant, and determines whether is in an early or late stage of development. Diagnosis may also include the removal and examination of lymph nodes to determine whether the cancer cells have spread.

Tumor markers are proteins found more often in the blood of individuals with the tumor than in normal individuals. These are not ideal compounds for diagnosing of cancer for two reasons. First, individuals without cancer may have elevated levels of the marker, leading to false positives. Second, tumor markers are not sufficiently elevated in all individuals with cancer to allow their detection. This leads to false negatives. One of the most commonly used tumor markers is **prostate-specific antigen (PSA)**. It is present in all adult males, but its level is increased after both benign and malignant changes in the prostate. Therefore, high levels of PSA indicate only that further tests are required to determine whether the condition is cancer. If prostate cancer is diagnosed, the levels of PSA can help to determine the effectiveness of treatment and detect recurrence. Another tumor marker is **CA125**, which is produced by a number of different cells, particularly ovarian cancer cells. It is used primarily to monitor the treatment efficacy of ovarian cancer. When the cancer is responding to treatment, CA125 levels fall. It is not used as a routine test for ovarian cancer because many common conditions that cause inflammation also increase the level of CA125, leading to a high incidence of false positives.

The earlier a cancer is found the more effectively it can be treated; however, early stage cancers typically produce no symptoms. Scientists are developing molelcular techniques to detect very early cancer. Using techniques such as mass spectrometry, they are also developing specific blood tests to identify a pattern of new proteins in the blood of individuals with a particular type of cancer. (See the *Proteomics* unit.) In addition, scientists are developing DNA microarrays to identify genes expressed in particular types of cancer cells. (See the *Genomics* unit.)

With the sequencing of the human genome and the mapping of **single nucleotide polymorphisms (SNPs)** (see the *Genomics* unit), it may be possible to diagnose particular cancers by identifying cells with known gene alterations. In 2002 scientists detected ovarian cancer by testing blood for the presence of DNA released by tumor cells. They looked for changes in certain alleles at eight SNPs that are characteristic of cancer. Using this technique, they successfully identified eighty-seven percent of patients known to have early-stage of ovarian cancer and ninety-five percent of those with late-stage ovarian cancer. The ability to determine which genetic alterations are associated with various cancers opens up the possibility of identifying cancerous cells while the cancer is in an early, treatable stage.

Traditional Treatments

Because cancer comprises many diseases, doctors use many different treatments. The course of treatment depends on the type of cancer, its location, and its state of advancement. Surgery, often the first treatment, is used to remove solid tumors. It may be the only treatment necessary for early stage cancers and benign tumors.

Radiation kills cancer cells with high-energy rays targeted directly to the tumor. It acts primarily by damaging DNA and preventing its replication; therefore, it preferentially kills cancer cells, which rapidly divide. It also kills some normal cells, particularly those that are dividing. Surgery and radiation treatment are often used together.

Chemotherapy drugs are toxic compounds that target rapidly growing cells. Many of these drugs are designed to interfere with the synthesis of precursor molecules needed for DNA replication; they interfere with the ability of the cell to complete the S phase of the cell cycle. Other drugs cause extensive DNA damage, which stops replication. A class of drugs called spindle inhibitors stops cell replication early in mitosis. During mitosis, chromosome separation requires spindle fibers made of microtubules; spindle inhibitors stop the synthesis of microtubules. Because most adult cells don't divide often, they are less sensitive to these drugs than are cancer cells. Chemotherapy drugs also kill certain adult cells that divide more rapidly, such as those that line the gastrointestinal tract, bone marrow cells, and hair follicles. This causes some of the side effects of chemotherapy, including gastrointestinal distress, low white blood cell count, and hair loss.

Newer Treatments

Many of the factors that affect normal cell growth are hormones. Although cancer cells have lost some of the normal responses to growth factors, some cancer cells still require hormones for growth. Hormone therapy for cancer attempts to starve the cancer cells of these hormones. This is usually done with drugs that block the activity of the hormone, although some drugs can block synthesis of the hormone. For example, some breast cancer cells require estrogen for growth. Drugs that block the binding site for estrogen can slow the growth of these cancers. These drugs are called selective estrogen receptor modulators (SERMs) or anti-estrogens. Tamoxifen and Raloxifene are examples of this type of drug. A ten-year clinical trial of these two drugs with 20,000 women began in 1999 to determine their effectiveness in preventing breast cancer. Similarly, testosterone (an androgen hormone) stimulates some prostate cancer cells. Selective androgen receptor modulators (SARMs) are drugs that block the binding of testosterone to these cancer cells, inhibiting their growth and possibly preventing prostate cancer.

Newer chemotherapeutic drugs target specific, active proteins or processes in cancer cell **signal transduction pathways**, such as receptors, growth factors, or kinases (see **Fig. 1**). Because the targets are cancer-specific proteins, the hope is that these drugs will be much less toxic to normal cells than conventional cancer drugs.

The oncogene *RAS* is mutated in many types of cancer, particularly pancreatic cancer, which has a poor rate of survival for those afflicted. The *RAS* protein is only active after it is modified by the addition of a specific chemical group. Scientists are developing drugs to inhibit the action of the enzyme that adds the chemical group to the *RAS* protein, resulting in an inactive form of *RAS*. Early tests indicate that these drugs show promise for reducing tumors in cancer patients.

A drug called Gleevec® inhibits cancer cell growth and causes cancer cells to undergo apoptosis, or programmed cell death. It binds to abnormal proteins in cancer cells, blocking their action in promoting

uncontrolled cell growth. Because it binds only to these abnormal proteins, Gleevec® does not show the high levels of toxicity of other chemotherapy drugs. Gleevec® was developed to treat a relatively rare cancer called chronic myeloid leukemia; however, it also appears to help other cancers.

Chemotherapy may fail because the cancer cells become resistant to the therapeutic drugs. One of the characteristics of cancer cells is a high frequency of mutation. In the presence of toxic drugs, cancer cells that mutate and become resistant to the drug will survive and multiply in the presence of the drug, producing a tumor that is also resistant to the drug. To overcome this problem, combinations of chemotherapy drugs are given at the same time. This decreases the probability that a cell will develop resistance to several drugs at once; however, such multiple resistances do occur. Some drug-resistant cancer cells express a gene called *MDR1* (multiple drug resistance). This gene encodes a membrane protein that can not only prevent some drugs from entering the cell, but can also expel drugs already in the cell. Some cancer cells make large amounts of this protein, allowing them to keep chemotherapy drugs outside the cell.

Another promising target for cancer therapy is angiogenesis. Several drugs, including some naturally occurring compounds, have the ability to inhibit angiogenesis. Two compounds in this class are angiostatin and endostatin; both are derived from naturally occurring proteins. These drugs prevent angiogenesis by tumor cells, restricting tumor growth and preventing metastasis. One important advantage of angiogenesis inhibitors is that, because they do not target the cancer cells directly, there is less chance that the cancer cells will develop resistance to the drug.

One contributing factor in cancer is the failure of the immune system to destroy cancer cells. Immunotherapy encompasses several techniques that use the immune system to attack cancer cells or treat the side effects of some types of cancer treatment. The least specific of these are the immunostimulants, such as interleukin 2 and alpha interferon, which enhance the normal immune response.

A technique called **chemoimmunotherapy** attaches chemotherapy drugs to antibodies that are specific for cancer cells. The antibody then delivers the drug directly to cancer cells without harming normal cells, reducing the toxic side effects of chemotherapy. These molecules contain two parts: the cancer-cell-specific antibody and a drug that is toxic once it is taken into the cancer cell. A similar strategy, **radioimmunotherapy**, couples specific antibodies to radioactive atoms, thereby targeting the deadly radiation specifically to cancer cells.

Another immunological approach uses antibodies that inactivate cancer-specific proteins, such as growth factors or tumor cell receptors, which are required by tumor cells. For example, many breast and ovarian cancer cells over-express a receptor protein called *HER2*. An antibody called Herceptin®, which binds *HER2*, inhibits tumor growth by preventing the binding of growth factors to these cells.

Some cancers, particularly leukemia, are treated with very high doses of chemotherapy drugs and radiation intended to kill all the cancer cells. The side effect of this harsh treatment is destruction of the bone

marrow, which contains stem cells. Stem cells, immature cells that develop into blood cells, are essential. After treatment, the patient's bone marrow must be restored, either from bone marrow removed from the patient before drug therapy or from a compatible donor. Although the patient's own bone marrow is best, it can contain cancer cells that must be destroyed before it is returned to the patient.

Table 2. Some Drugs Used in the Treatment of Cancer

CLASS	MECHANISM
selective estrogen receptor modulators (SERM) (Tamoxifen and Raloxifene)	blocks the binding site for estrogen; can slow the growth of estrogen-stimulated cancers
selective androgen receptor modulators (SARM)	blocks the binding site for testosterone; can slow the growth of testosterone-stimulated cancers
spindle inhibitors	stops cell replication early in mitosis
farnesyl transferase inhibitors	blocks the addition of a farnesyl group to RAS, preventing its activation
Gleevec®	binds to abnormal proteins in cancer cells, blocking their action
angiogenesis inhibitors (endostatin, angiostatin)	prevent angiogenesis by tumor cells
immunostimulants (interleukin 2, alpha interferon)	enhance the normal immune response
Herceptin®	antibody that binds to *HER2* receptor on tumor cells, preventing the binding of growth factors

Preventing Cancer

Cancer appears to result from a combination of genetic changes and environmental factors. A change in lifestyle that minimizes exposure to environmental carcinogens is one effective means of preventing cancer. Individuals who restrict their exposure to tobacco products, sunlight, and pollution can greatly decrease their risk of developing cancer. Many foods contain antioxidants and other nutrients that may help to prevent cancer. The National Cancer Institute recommends a diet with large amounts of colorful fruits and vegetables. These foods supply ample amounts of vitamin A, C, and E, as well as **phytochemicals** and other antioxidants that help to prevent cancer. There is strong evidence that a diet rich in vegetables and fruits will not only reduce the risk of cardiovascular disease, obesity, and diabetes, but will also protect against cancer.

Vaccines also offer some promise for prevention of cancer. The first vaccine to prevent cancer was for hepatitis B, which is associated with liver cancer. An effective hepatitis B vaccine is available that can prevent both hepatitis and the cancer that may follow this infection. In 2002, test results of a papillomavirus vaccine were reported. Human papillomavirus type 16 infects about twenty percent of adults. Although most papillomavirus infections do not cause cancer, some are associated with cervical cancer. A vaccine against this virus was administered to 1,200 young women in the United States. Within eighteen months, the vaccine produced high levels of antibodies to the virus, and prevented both papillomavirus infection and precancerous lesions in all the women. In the control group of about 1,200 women who did not receive the vaccine, forty-one infections and nine precancerous lesions were found. The vaccine can also prevent genital

warts caused by this virus strain. It appears that vaccines such as these may help in the fight to prevent cancers associated with viruses.

Screening, Genetic Tests and Counseling

Early diagnosis of cancer greatly increases survival; therefore, regular exams for cancer can help to prevent deaths from cancer. These include mammograms and Pap tests for women, prostate cancer tests for men, colonoscopy exams for colon cancer, and regular physical exams for other types of cancer. Individuals with a strong family history of cancer should consider genetic tests for cancer and cancer risk counseling. The focus of cancer risk counseling is the individual's personal risk of developing cancer and appropriate actions based on that risk.

The discovery of the *BRCA1* and *BRCA2* genes associated with early development of breast cancer has allowed women with a family history of early breast cancer to be tested for mutations in these genes. Only five to ten percent of breast cancers show evidence of inheritance. Of these, forty-five percent are associated with a mutation in *BRCA1* and thirty-five percent with *BRCA2*. The gene or genes for the remaining twenty percent are not yet known. If the *BRCA1* and *BRCA2* test results are negative, there is no evidence that the woman will have breast cancer because of these mutations. However, she may get breast cancer because of somatic mutations in these or other genes. If the *BRCA1 or BRCA2* test is positive, other family members may be tested to determine whether the gene was inherited. If other family members are negative, then there is less chance of hereditary risk of this form of cancer, although the individual with the mutation does carry an increased risk of the disease. If the test is positive in other family members, there is an increased hereditary risk for breast cancer in that family. The absence of hereditary risk does not mean that there is no other risk for breast cancer.

Decisions based on genetic tests can be very complicated. Individuals must be fully informed about the risks before they can make reasonable decisions. Genetic counselors are trained to help individuals make difficult decisions based on genetic tests. The cumulative risk of breast cancer to age seventy for a woman with a *BRCA1* mutation is about fifty-seven to eighty-five percent depending on whether she is in a high-risk family. Some women find the fear of cancer so disruptive to their lives that they choose mastectomy to prevent cancer. (This is called prophylactic mastectomy.) Similarly, women with *BRCA1* have a high lifetime risk of ovarian cancer, causing some of them to choose to have their ovaries removed. While these are difficult decisions, the availability of genetic information provides individuals with information that they can use to make such important medical decisions. A young woman with a strong family history of ovarian cancer might find by genetic testing that she does not have the *BRCA1* mutation and should not consider removal of her ovaries.

Further Reading

Book

Schneider, K. 2001. *Counseling about cancer: Strategies for genetic counseling.* 2d ed. New York: John Wiley & Sons.
Excellent information on the causes of cancer, genetic tests for predisposition to cancer, ethics, and genetic counseling.

Articles

Bahls, C., and M. Fogarty. 2002. Reining in a killer disease. *The Scientist* 16[11]:16.
An outline of several approaches to controlling cancer.

Gibbs, W. Wayt. 2003. Untangling the roots of cancer. *Scientific American,* July, 57–65.
New evidence challenges old theories of how cancer develops.

Kling, Jim. 2003. Put the blame on methylation. *The Scientist* 16[12]:27–28.
Gene silencing by methylation rather than by gene mutation may create some cancer cells.

McCook, A. 2002. Lifting the screen. *Scientific American,* June, 16–17.
Developing protein "fingerprints" to screen for cancer.

Rayl, A. J. S., and Lewis, R. 2001. In cancer research, diet and exercise roles strengthen. *The Scientist* 15[20]:17.
Evidence for the importance of lifestyle in preventing cancer.

Veggeberg, S. 2002. Fighting cancer with angiogenesis inhibitors. *The Scientist* 16[11]:41.
Discussion of a class of drugs that helps to prevent angiogenesis.

Wilson, J. F. 2001. A dual role for CDK inhibitors. *The Scientist* 16[6]:20.
Discusses approaches to cancer treatment using cells' cycle inhibitors.

Wilson, J. F. 2002. Elucidating the DNA damage pathway. *The Scientist* 16[2]:30.
How researchers have deciphered the role of DNA damage repair in cancer.

Glossary

Anaplastic. A term used to describe cancer cells that divide rapidly and have little or no resemblance to normal cells.

Angiogenesis. Blood vessel formation. Tumor angiogenesis is the growth of blood vessels from surrounding tissue to a solid tumor. This is caused by the release of chemicals by the tumor.

Apoptosis. A normal series of events in a cell that leads to its death. Also called "programmed cell death."

CA125. A substance sometimes found in an increased amount in the blood, other body fluids, or tissues that may suggest the presence of some types of cancer.

Chemoimmunotherapy. Chemotherapy combined with immunotherapy. Chemotherapy uses different drugs to kill or slow the growth of cancer cells; immunotherapy uses treatments to stimulate or restore the ability of the immune system to fight cancer.

Cyclin-dependent kinases. Proteins that add a phosphate to a number of proteins that control steps in the cell cycle.

Cyclins. Proteins that form complexes with cyclin-dependent kinases to control various steps in the cell cycle.

Dysplasia. Cells that look abnormal under a microscope but are not cancerous.

Hyperplasia. An abnormal increase in the number of cells in an organ or tissue.

Kinase. An enzyme that catalyzes the transfer of a phosphate group from ATP to another molecule, often a protein.

Oncogene. An altered form of a gene that normally directs cell growth. Oncogenes can promote or allow the uncontrolled growth of cancer. Alterations in a proto-oncogene, resulting in an oncogene, can be inherited or caused by an environmental exposure to carcinogens. (See *proto-oncogene*.)

Phytochemicals. Chemicals found in plants. Many of these chemicals are thought to reduce a person's risk of getting cancer.

Proto-oncogene. A gene that normally directs cell growth; if altered it may become an oncogene.

Prostate-specific antigen (PSA). A substance produced by the prostate that may be found in an increased amount in the blood of men who have prostate cancer, benign prostatic hyperplasia, or infection or inflammation of the prostate.

Radioimmunotherapy. Treatment with a radioactive substance that is linked to an antibody that will attach to the tumor when injected into the body.

Signal transduction pathway. A series of events controlled by signal molecules that bind to membrane proteins. These, in turn, activate cytoplasmic proteins, which ultimately activate transcription factors.

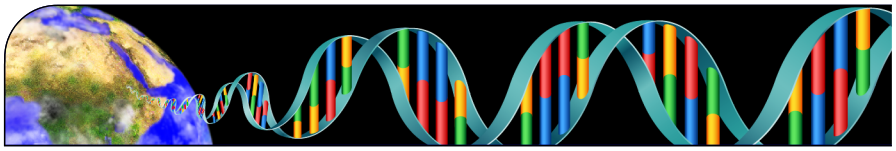
Single nucleotide polymorphism (SNP). Variations in the DNA sequence that occur when a single nucleotide (A, T, C, or G) in the genome sequence is changed.

Telomerase. An enzyme that replaces the repeat sequences at the ends of chromosomes that are lost during chromosome replication.

Telomeres. The ends of chromosomes containing repeat sequences; these ends are shortened each time the chromosome replicates.

Transcription factor. A protein that influences transcription of another gene by binding to DNA.

Tumor suppressor gene. Genes that can suppress or block the development of cancer.



REDISCOVERING BIOLOGY

Molecular to Global Perspectives

Human Evolution

"...we come from a long line of failures. We are apes, a group that almost went extinct fifteen million years ago in competition with the better-designed monkeys. We are primates, a group that almost went extinct forty-five million years ago in competition with the better-designed rodents. We are chordates, a phylum that survived in the Cambrian era 500 million years ago by the skin of its teeth in competition with the brilliantly successful arthropods. Our ecological success came against humbling odds." M. Ridley [1]

We humans have always held a special fascination with our place in the evolutionary pageantry. Where did we come from? That we share close common ancestry with the apes is not in doubt. But what kind of an ape are we? How long ago did our lineage separate from the other apes? Are we still evolving? What can molecular genetics tell us about our history and our future?

Concerning our place among the apes, Thomas Huxley (known as "Darwin's bulldog" because of his popularization of Darwin's theory of evolution) provided an early correct answer in 1863. Huxley placed humans with chimpanzees and gorillas (the great apes of Africa), and

Courtesy of Roger Birkell.

Courtesy of Roger Birkell.

Figure 1. A gorilla (left) and a chimpanzee (right), our closest living relatives.

separate from orangutans and gibbons. Numerous lines of evidence have since strongly supported this view. Morphologically, we share many derived traits with the African apes, including enlarged brow ridges, elongated skulls, shortened canine teeth, and enlarged mammary glands. We are also much more similar at the DNA level to the African apes than we are to any other species.

Scientists have identified three species of African apes: the gorilla *(Gorilla gorilla)* and two species of chimpanzees. *Pan troglodytes,* the common chimpanzee, is the larger of the two. *Pan paniscus,* which had previously been called the "pygmy chimpanzee," is now named the "bonobo." In addition to size, the two chimpanzees differ in social structure and temperament; the bonobos appear to be more peaceful and egalitarian than the common chimpanzees. Both species of chimpanzees use tools; however, the types of tools vary in different populations of both species.

By about 1980 a combination of crude molecular techniques — based on the divergence of certain proteins and the fossil record — allowed us to determine that humans, chimps, and gorillas last shared a common ancestor approximately five million to eight million years ago. Those methods, however, were not able to ascertain the order in which the three species split. Several questions remained. For instance, are chimpanzees and gorillas each other's closest relatives? Or is the closest relationship between humans and chimpanzees? Or is it between humans and gorillas?

We could encapsulate the outdated view of evolution as progress up a ladder of changes. First the ability to walk upright (bipedalism) appeared. Soon after, the lineage leading to humans (the **hominids**) split off from the other African apes. Many fossils of the genus *Australopithecus* demonstrate that the earliest bipedal hominids did not substantially differ from chimpanzees in brain size. In the outdated view of human evolution, there was a slow steady increase in brain size as *Australopithecus afarensis* (better known as "Lucy") evolved into *Homo habilis*, and then into *Homo erectus.* Brain size continued to increase until the appearance of *Homo neanderthalis* (the Neanderthals), who looked much like us but had larger brow ridges.

This older view of human evolution is not so much incorrect as it is incomplete and misleading. New fossil evidence demonstrates that the hominid lineage, our family tree, is more bush-like than ladder-like. Studies of these fossils show that several species of hominids coexisted for long periods of time. New molecular genetic evidence allows us to address which two of the three species — chimpanzee, gorilla, and human — represents the two closest relatives. Genetic data also can address the patterns of variation within and among human population. In addition, the molecular genetic data demonstrate how infectious disease has shaped genetic variation in humans.

New Fossils

During the 1990s archaeologists unearthed dozens of new fossil hominids. These have been particularly useful for illuminating the changes that took place as the human lineage split from the chimp lineage. One important find was in Ethiopia by Tim White (University of California-Berkeley), Gen Suwa (University of Tokyo), and others who found a fossil, which they determined to be 4.4 million years old. The fossil, *Ardipithecus ramidus*, probably represents a transitional form with respect to the evolution of bipedalism: while it may have been able to walk upright, it had a different posture than we do. It probably spent some time upright and some time walking like a chimp, on its knuckles. In other respects, it looked much like chimp, except for

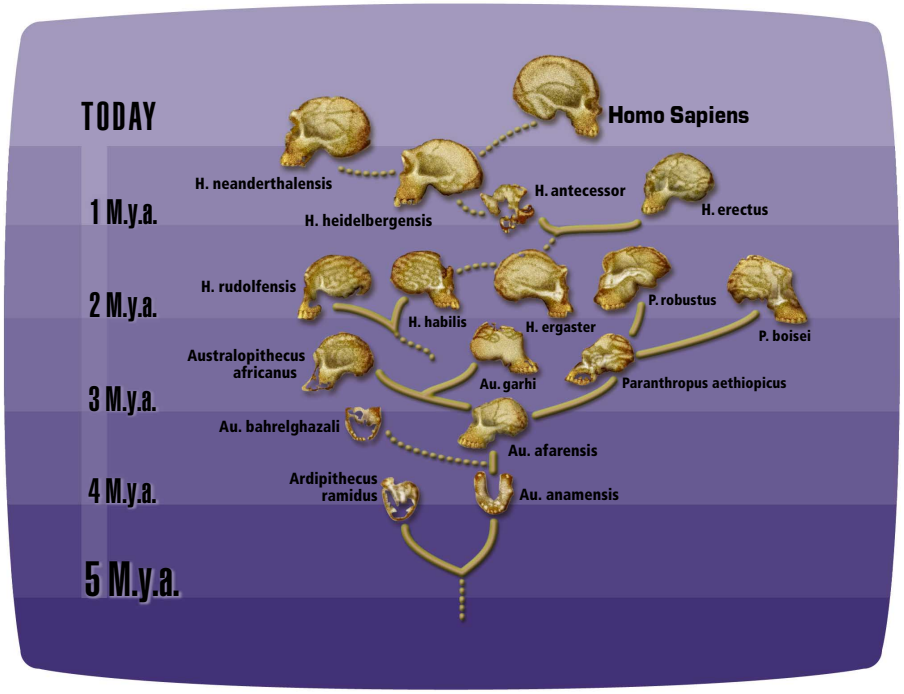


Photo-illustration — Bergmann Graphics

Figure 2. The human "bush," as postulated from fossil finds of hominid species.

subtle differences in teeth and skull. The first, clearly bipedal hominids — *Australopithecus anamensis* and *Australopithecus afarensis* — appeared about 4.1 million years ago, shortly after *A. ramidus*.

Other fossil discoveries illustrate the bushiness of the human lineage. As seen in the illustration, as many as four different apparent species often lived at the same time (**Fig. 2**). While there was a general trend toward increased brain size with time, species with considerably different brain sizes lived simultaneously. Questions remain about how different species replaced previous ones. Was it through warfare? Was it that the replacing species were better competitors? Perhaps it was simply a random event. We don't really know.

Despite the inferences we can draw from these new fossil findings, the fossil record still has limitations; it is incomplete. How can one determine whether different fossils belong to the same species? Species determinations are based on the ability, or the perceived ability, of different groups to interbreed. In cases where it is infeasible or immoral to do experiments crossing the two groups, one can infer the capacity for the groups to interbreed based on genetic data. Yet, with few exceptions, scientists cannot extract DNA evidence from fossils; only morphological characters are available. How then can one make the inferences about the capacity to interbreed? For instance, sexual dimorphism may lead one to classify males and females of the same population as separate species.

What Does DNA Tell Us About Our Position Among the Apes?

The new genetic data have substantially contributed to our understanding of the relationship between our species and its closest relatives. Based on several independent lines of evidence, we can now say with confidence that humans are more related to chimpanzees than

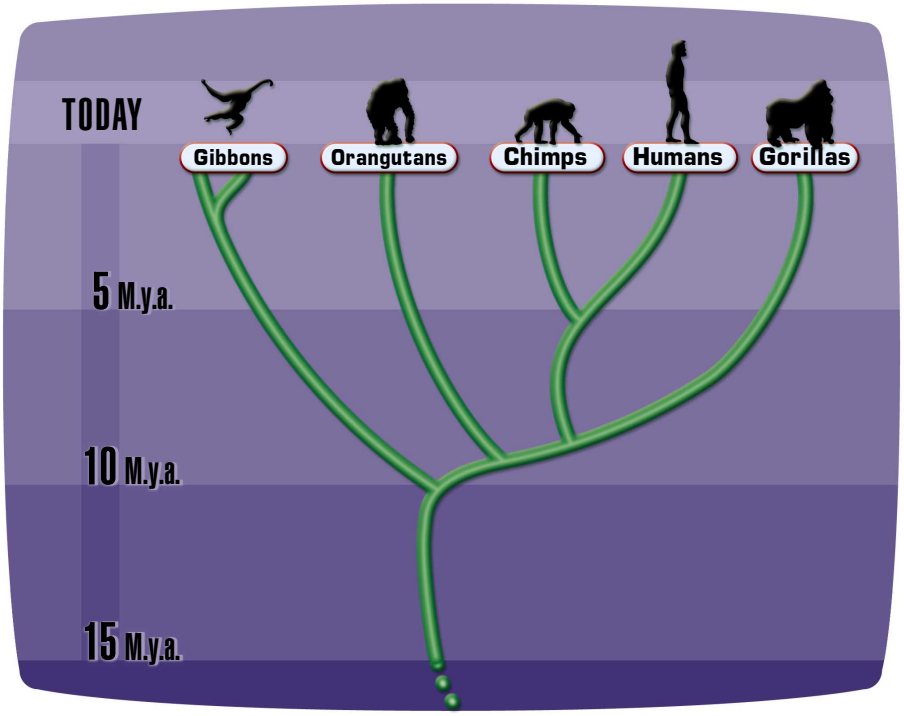


Photo-illustration — Bergmann Graphics

Figure 3. A tree showing the evolution of the hominoids, including the great apes and humans.

to gorillas (**Fig. 3**). While the two species of chimpanzees are each other's closest relatives, their next closest relative is *H. sapiens* and not *G. gorilla.*

How do we know this? Evolutionary geneticists have been increasingly able to draw better and more robust inferences about the relationships among different organisms based on morphological and molecular genetic data, and new systematic methodology. These methods have also been used to determine our relationship among the apes. (See the *Evolution and Phylogenetics* unit.) In essence, groups of organisms (known as **taxa**) are placed into **clades** that are nested in larger clades based on shared ancestry. All of the taxa in a given clade are assumed to have a single common ancestor.

The first DNA-based data used to determine the relationships of the African apes came from mitochondria. These intracellular organelles enable animals to use aerobic respiration and have DNA that evolves relatively quickly in mammals. Consequently, mitochondrial DNA (mtDNA) is useful in analyzing the relationships of closely related species and populations within species. Mitochondria are also abundant in cells and, thus, mtDNA was easier to obtain than nuclear DNA.

New DNA amplification technologies developed during the 1990s, such as the polymerase chain reaction (PCR), makes obtaining sufficient quantities of DNA much easier. (See the *Genetically Modified Organisms* unit.) Yet, for historical reasons, most taxonomic studies that used DNA characters were first done with mtDNA. Based on the evidence from mtDNA sequences, chimpanzees and humans were determined to be each other's closest relatives. These studies further suggest that humans and chimpanzees separated almost five million years ago, and the human-chimp clade separated from gorillas almost eight million years ago.

Critics raised an important point about the inferences based on the mtDNA studies: it is based on only a single, independently evolving gene region. When one considers very closely related groups of species, the constructed phylogenetic tree based on data from one gene may be different than one constructed from a different gene. Either one or both **gene trees** may not accurately reflect the true evolutionary history of the species. This phenomenon occurs because of genetic variation (**polymorphism**) in the ancestral species. Ancestral polymorphism can segregate differently in the different descendant species; that is, in one of the different descendant species, one of the variants may become fixed and in another descendant species a different variant may be fixed. Either natural selection or random genetic drift can cause this phenomenon. In either case, there is possibility that the history of the gene region may not reflect the history of the species. In other words, suppose that chimps really did split first from the lineage containing humans and chimps. It would still be possible that the phylogenetic tree based on a single gene may have gorillas splitting off from humans and chimps, or humans splitting from chimps and gorillas.

In the case of determining the relationships among the African apes, the solution to this challenge was simply the collection of more data from more genes. Mary-Ellen Ruvolo analyzed data sets from fourteen independent gene regions. In eleven of the cases, humans and chimps are each other's closest relatives (**sister taxa**). In two cases, gorillas and chimpanzees are sister taxa, and in one case humans and gorillas are sister taxa. Statistical tests show that these results are highly unlikely to arise unless humans and chimpanzees are indeed each other's closest relatives. Subsequent analyses with even more genes have corroborated the conclusion reached by Ruvolo and the earlier mitochondrial DNA studies: we are closest to chimps.

Variation Within and Among Human Populations

At the DNA level, humans are both very similar to and very different from one another. On average, pairs of individual humans share 99.9% DNA sequence identity. Due to the sheer size of our genomes, however, we possess numerous differences from one another. The human genome consists of just over three billion nucleotides; that 0.1% of difference represents three million variants between the average pair. The vast majority of these variants have no functional significance. However, even if one in a thousand did, that would still mean that we would each differ at thousands of functionally important sites.

How does this variation compare with that of other species? Humans actually have less genetic variation than do their closest relatives. For instance, the average difference between two randomly selected chimpanzees is roughly four times greater than between two humans. This is, at first glance, surprising. Based on population genetic theory, levels of genetic variation within species should correlate positively with population size. This predicted correlation comes about because the strength of random genetic drift — which results in the loss of genetic variation — increases at lower population sizes. Yet, the human population numbers in the billions, and the population sizes of chimpanzees and gorillas is fewer than a hundred thousand.

What could explain that discrepancy? The strength of genetic drift is dependent not on the current census population size but on the historical population sizes. The relatively low levels of genetic variation in humans can be explained by a severe, but short-lasting, population bottleneck, where the population of our species was likely reduced to a few thousand. It could also be explained by a more moderate, sustained bottleneck. During this bottleneck the population was possibly in the tens to hundreds of thousands for a more considerable time. Alternately, natural selection could also either increase or decrease the extent of variation in one of the species. Yet, because it is unlikely that natural selection would act in the same way on multiple regions of the genome, the difference in the extent of genetic variation between humans and chimpanzees is more likely a consequence of historical demography.

How is this variation partitioned according to known racial groups? During the 1970s the then state-of-the-art technique of electrophoresis of protein variants showed that around eighty to ninety percent of human genetic variation was within ethnic populations, five to ten percent was among ethnic populations within the major racial groups, and only about five to ten percent was among the major racial groups. In other words, "if everybody on earth became extinct except for the Kikiyu of East Africa, about eighty-five percent of all human variation would still be present in the reconstituted species"[2]. More recent analyses of DNA sequence data strongly confirm the results of earlier protein electrophoresis studies. In both the protein electrophoresis and the DNA sequence studies, the differences between racial groups are generally ones of frequencies and not kind. The situation of "fixed differences" — in which all individuals in one group have variant A and all individuals of another group have variant B — is extremely rare in humans. Instead, groups vary by having different frequencies of genetic variants. There are cases of "private alleles," however, where genetic variants are found in low to intermediate frequencies in some populations but are virtually absent from others.

Out of Africa?

As with determining the relationships of the apes, the first DNA-based studies of the relationships of human populations also used mitochondrial DNA. In mammals, mitochondria have an interesting inheritance pattern: they are transmitted nearly exclusively along maternal lines. Although males have mitochondria, they do not transmit them to their offspring. Thus, all of your mitochondria came from your maternal grandmother and, by extension, your maternal-maternal great-grandmother. In 1987 Rebecca Cann, Mark Stoneking, and Alan Wilson (then at University of California-Berkeley) published a controversial and provocative paper in *Nature*, stating that they had located the common ancestor of all mitochondrial variants — the so-called **Mitochondrial Eve** (**Fig. 4**). They placed her in Africa approximately 200,000 years ago; subsequent studies have found similar results. Fifteen years after that first paper the results remain a source of interest and controversy.

Why are the Mitochondrial Eve studies a continual source of controversy within the human evolutionary genetics community? That there is a common ancestor of mitochondrial DNA sequences is not a surprise. In fact, it is a consequence of Mendelian genetics: genes taken

from any sample within a population will share a common ancestor. Take pairs of gene copies in the same population. Some of them will share the same ancestor from one generation ago; they are from siblings of the same parents. Some pairs of gene copies will trace a common ancestor two generations back. Some pairs will share common ancestry even further back. However, eventually, all copies will share a common ancestor. What is of interest is how long it takes all gene copies to coalesce to that ancestor.

What the debate focuses on is the timing and the location of the Mitochondrial Eve. The initial studies showed that there was one clade consisting only of African individuals, and one with African and other individuals. Hence, we can infer that the common ancestor lived in Africa. Numerous researchers challenged the methodology of the original study. For instance, the original study used African-American individuals instead of individuals from Africa. Most of the subsequent studies, using more data — including data from individuals from several African tribes — and better methodology seem to confirm that Africa is the location of the common ancestor.

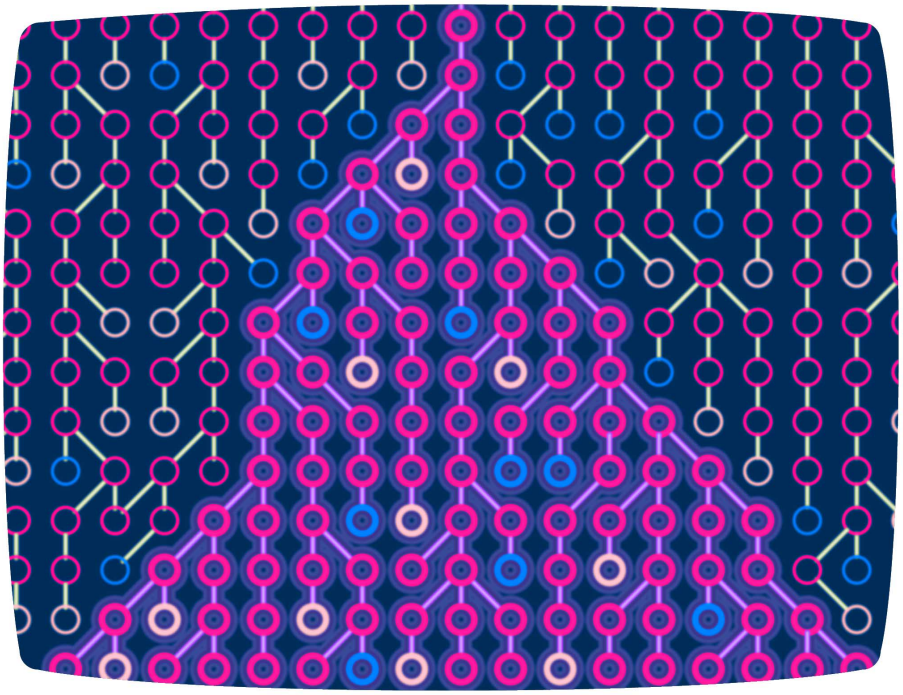


Illustration — Bergmann Graphics

Figure 4. The diagram illustrates how one line of mitochondrial DNA came to be carried by all living humans, passed down to us through the "Mitochondrial Eve."

To determine the age of the Mitochondrial Eve, biologists need to first make assumptions about the way evolution proceeds. The usual assumption is that changes in the DNA occur roughly in a clock-like fashion — that there is a so-called **molecular clock**. The molecular clock assumes that groups separated by twenty nucleotide changes have common ancestors that are roughly twice as old those separated by ten nucleotide changes. No one believes DNA evolution proceeds in a perfect clock-like manner. What is debated is the extent to which the clock assumption can provide an estimate about divergence times. The usefulness and the accuracy of molecular clocks have been controversial ever since Zuckerkandel and Pauling proposed them in the 1960s. Yet, most evolutionary biologists agree that the molecular clock concept has at least some validity.

Inferring dates based on a molecular clock also requires that one calibrate the clock. How quickly do the changes occur in the lineage(s) of interest? Different molecular clocks based on different regions of the genome or different types of organisms don't all tick at the same rate. To calibrate a molecular clock, researchers usually use a lineage split for which they have at least some degree of confidence about when it occurred. They then divide the amount of genetic divergence by the time when the groups last shared a common ancestor. In the case of mtDNA, the calibration was set by the human-chimp split of six million years. Because chimps and humans differ by about twelve percent of nucleotides in mtDNA, the rate of change for the hominid lineage for mtDNA is about two percent per million years. The average total divergence from contemporary sequences to the inferred sequence of the mtEve is about 0.4% and, thus, the divergence time is about 200,000 years. The confidence limits, however, of this estimate are rather large. Christopher Wills once concluded that it is possible that the upper-end of mtEve's age may be as much as 800,000 years; new data places his latest estimate at 400,000 years.[3]

Different genes will often have different evolutionary histories. One should not expect the male equivalent ("the Y chromosome Adam") to have lived at the same place and the same time as the Mitochondrial Eve. Owing in part to having a lower mutation rate, the human Y chromosome generally has less variation than the mitochondria, which makes analysis more difficult. Nonetheless, recent studies suggest that the last common ancestor of all existing human Y chromosomes also lived in Africa — but more recently than Mitochondrial Eve.

Largely from the Mitochondrial Eve studies, one model — the **out of Africa** hypothesis — gained favor among anthropologists and human evolutionary geneticists. This hypothesis, which is sometimes called the "replacement hypothesis," postulates that modern *Homo sapiens* spread out of Africa, into Europe and Asia, and replaced archaic *Homo sapiens* living in those regions (**Fig. 5**). In contrast, Milford Wolpoff and others have proposed the **multiregional hypothesis**. They argue that the archaic *Homo sapiens* populations in the different regions (Europe, Asia, and Africa) all evolved together into modern *Homo sapiens*. While genetic changes would first occur in one locality, gene flow would spread those changes into the other localities.

The out of Africa and multiregional hypotheses make several distinct predictions. One would predict that under the out of Africa hypothesis, Africa would be the origin of the common ancestor of variants for most of the independent data sets (different genes) tested. The multiregional hypothesis would predict a random pattern. Under the out of Africa model, the divergence time between the African and the non-African populations would have an upper-limit of about 200,000 years. In contrast, the multiregional hypothesis would predict a divergence time of approximately one million years. One caveat is that the apparent age of the divergence could be reduced by the gene flow among the populations. Another caveat is that selection can also alter the apparent divergence times. The out of Africa hypothesis also predicts that there will be more genetic diversity within the African population than within the other populations.

As of 2003 the evidence seems to favor the out of Africa model though some intermediate positions cannot be ruled out. In nearly all of the studies more genetic diversity is seen in the African populations than in

Figure 5. **Top**: The "out of Africa," or "replacement," hypothesis suggests all living humans evolved from a group that originated in Africa. **Bottom**: The "multiregional" hypothesis suggests several groups evolved in parallel to form today's population of humans.

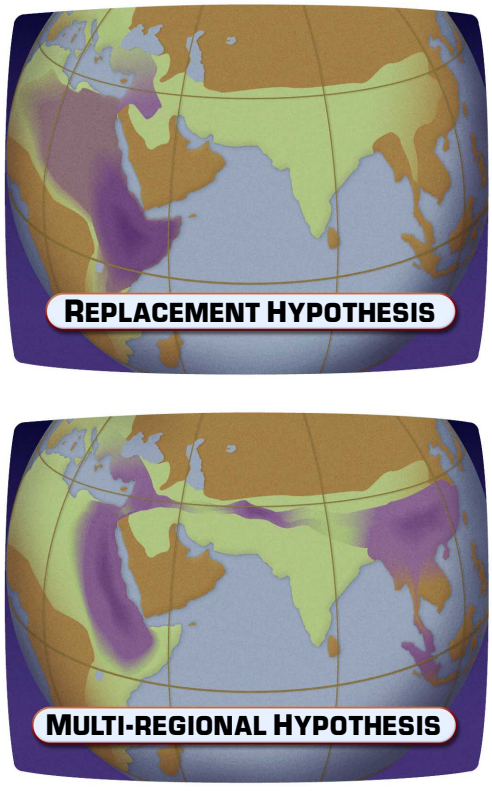


Photo-illustration — Bergmann Graphics

others. In addition, the divergence times appear more consistent with the out of Africa than the multiregional hypothesis. As we obtain more and more sequences from different regions from the genome, this debate should become resolved.

Neanderthals in Our Gene Pool?

Have Neanderthals contributed to our gene pool? This question is related to, but is distinct from, the "out of Africa" debate. If Neanderthals had made a substantial contribution to the gene pool of contemporary humans, replacement models like out of Africa would be severely challenged. On the other hand, while the lack of Neanderthal contribution to the contemporary human gene pool would be consistent with the out of Africa model, that particular result alone would not disprove the multiregional hypothesis. It is also possible that there was substantial exchange of genes across many different human populations but that the Neanderthal population was not involved.

How can we tell whether Neanderthals contributed to the contemporary gene pool? You can't get DNA from fossil humans. Or can you? Data from fragments of DNA collected from different Neanderthal fossils have led to the conclusion that Neanderthals probably did not contribute to the contemporary gene pool. In 2000 Igor Ovchinnikov and his colleagues were able to obtain small fragments of mtDNA from a 29,000-year-old Neanderthal fossil found in the Caucasus Mountains. They compared the mitochondrial sequences from their fossil to mtDNA collected from a previously collected Neanderthal fossil from Germany. Ovchinnikov and his collegues concluded "Phylogenetic analysis places the two Neanderthals from the Caucasus and western Germany together in a clade that is distinct from modern humans, suggesting that their mtDNA types have not contributed to the modern human mtDNA pool."[4]

Human Genetic Variation and Disease

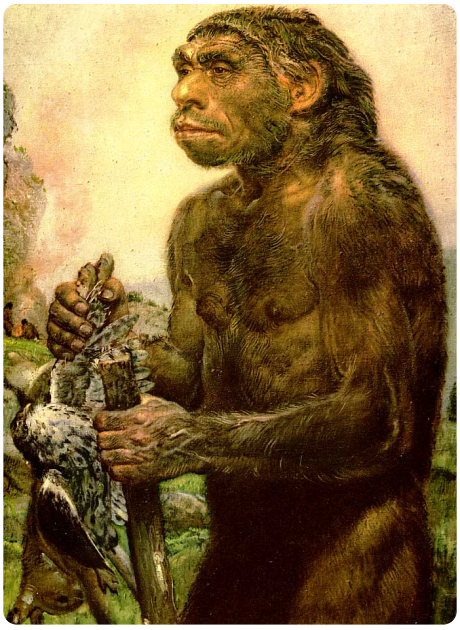
Disease has continued to have a strong impact on human mortality and reproduction. One would expect there to be genetic variation for the ability to resist disease. Indeed, there is such variation. Moreover, biologists have been increasingly able to correlate variation at specific genetic loci, and susceptibility to or severity of various diseases. For example, scientists are combining genetic and genealogical data to locate genes that affect disease tendency in Icelanders.

Genetic variation for disease resistance and natural selection associated with disease has shaped the evolution of our species. Below, we discuss the impact of two infectious diseases: malaria and HIV. We conclude with a discussion of the genetics of asthma propensity — an illustration of the complex interplay of genetics and environmental effects.

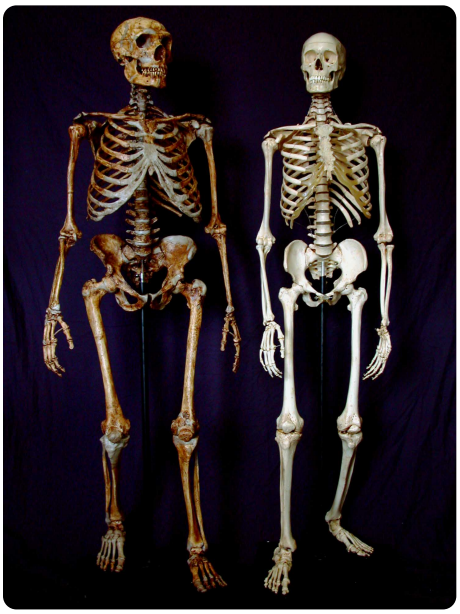
Malaria, Sickle Cell Anemia, and Balancing Selection

Sickle cell anemia affects approximately 70,000 Americans, almost exclusively those with African ancestry. The lifespan of an individual with sickle cell anemia is currently approximately 40 years in the United States. Before the advent of modern medicine, individuals with the disease usually died before they could have offspring.

Figure 6. Top: An artist's rendering of a Neanderthal man. Bottom: An adult human (right) and Neanderthal skeleton (left) side by side.



Zdenik Burian, Neanderthal (1960). Courtesy of the Moravian Museum.



Courtesy of the American Museum of Natural History

The disease is caused by a change in a single amino acid difference in the beta chain of hemoglobin. Individuals with two copies of the sickle form of the gene have sickle cell anemia. Heterozygotes — individuals with one normal and one mutant copy of the gene — appear normal and do not manifest the disease except under very stressful conditions; however, they are carriers. If two carriers have a child, the child has a twenty-five percent probability of receiving two copies of the sickle form and having the anemia. Approximately ten percent of African Americans are carriers. In Africa itself the frequencies of the disease and carriers are even higher.

If sickle cell anemia is so deadly, why are so many people heterozygous carriers of the disease? Moreover, why does the disease afflict predominantly one racial group? Surpisingly, the answer has to do with malaria. Heterozygote sickle cell carriers are much more resistant to malaria than those with just normal hemoglobin. Because heterozygotes have the best of both worlds (no sickle cell anemia and higher malaria resistance) and malaria is extremely prevalent in Africa, the sickle allele can be maintained in balance with the normal allele. Note that in the United States, where malaria is rare, the carriers possess no such advantage and may even have a small selective disadvantage. Therefore, due to the strong selection acting against those with the anemia, the frequency of sickle cell anemia should slowly decline in the United States. That the frequency of the sickle cell allele is higher in African populations than in African-Americans is due to both this selection and the genetic mixing between whites and blacks in the United States.

This situation, where selection actively maintains two or more alleles at a locus, is called **balancing selection.** Balancing selection can arise by the heterozygotes having a selective advantage, as in the case of sickle cell anemia. It can also arise in cases where rare alleles have a selective advantage. In extreme cases, balancing selection can maintain alleles in populations long enough for speciation to occur. In such cases, one species may have alleles that are more similar to those of the other species than they are to other alleles of the same species. One case of this phenomenon occurs at loci at the major histocompatibility complex (MHC) wherein some human alleles are much more closely related to some chimpanzee alleles than they are to other human alleles (**Fig. 7**). MHC — also called the human leukocyte antigen (HLA) loci when referring to it in humans — encodes proteins that are used to recognize foreign invaders by cells of the immune system. Chimp-like alleles have been maintained in the human population not because they are chimp-like, but because either having rare alleles or having two different alleles has provided a selective advantage. This balancing selection is so powerful that alleles are maintained that predate the human/chimp split.

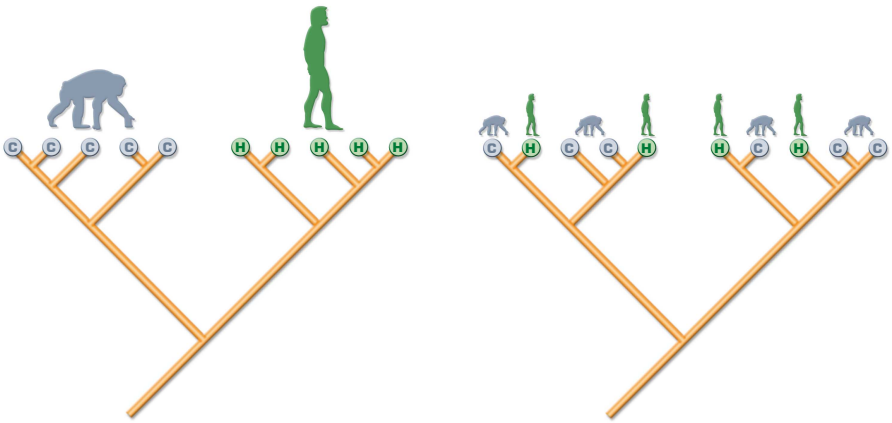


Figure 7. For nearly all genes, human alleles cluster together and chimp alleles cluster together (left). In the case of the major histocompatibility complex (MHC), human alleles are often more closely related to chimp alleles and vice-versa. This occurs due to balancing selection maintaining variation at the MHC (right).

Illustration — Bergmann Graphics

Resistance to HIV

Despite the lethality of HIV/AIDS, susceptibility to HIV infection and progression to AIDS is rather variable. There are individuals who have been exposed to HIV multiple times but who either remain uninfected or if they are infected, progress more slowly to full-blown AIDS. Recent studies have shown that some of the variation in HIV resistance has a genetic component.

HIV operates by subverting the immune system; therefore, it is logical that differences in the immune system may play a role in the genetic variation of resistance to HIV. Indeed, some HIV-resistant individuals possess different **chemokine receptors** than HIV-susceptible individuals. What's a chemokine receptor? First, let's discuss chemokines.

Chemokines are molecular signals released by cells of the immune system that stimulate white blood cells to move to inflamed tissues. They are metaphoric "cries for help." The chemokines bind to receptors located on the white blood cells. Macrophages — those white blood cells that engulf foreign particles and are an early stage of defense — possess the chemokine receptor that is encoded by the gene CCR5. By subverting the normal function of this chemokine receptor, HIV is able to gain entry into macrophages. (See the *HIV and AIDS* unit.)

Individuals that have lower expressions of this protein due to variants of the CCR5 gene have an increased resistance to HIV; their macrophages are metaphorically more cautious about the signals they respond to. The most obvious case of a "more cautious" CCR5 variant is the allele that has a deletion of thirty-two nucleotides. Individuals who are heterozygous for this variant, CCR5-delta32, have substantially increased resistance to HIV infection; if infected, progress to full-blown AIDS is much slower than normal. Individuals that are homozygous for CCR5-delta32 are virtually completely resistant to HIV. In European populations about twenty percent of individuals are heterozygotes, and one percent are homozygotes in some populations. In contrast, the allele is rare in the Asian populations and virtually absent in the African populations.

Why is this deletion variant present in some populations in such high frequencies? HIV is, at most, a couple centuries old and, more likely, less than a hundred years old. That isn't sufficient time for natural selection to increase the frequency of a rare allele, such as is observed in the European populations. Furthermore, the selection pressures

caused by HIV should be much higher in Africa than in Europe. It is also probable that the decreased receptivity to chemokines would be somewhat costly. Some biologists have suggested that the deletion allele could be a vestige of plague resistance. It may have led to increased survival during the Black Plague of the fourteenth century in Europe, and has had an unintended — but welcome — consequence of HIV resistance. The increased frequency of the variant in Europe would be consistent with that scenario.

The environment and, in particular, disease, has continued to exert strong pressures on human populations. Generally, we are unable to directly observe changes in species because these changes occur in time scales that exceed human lifespans. Yet, we may be able to detect small changes in allele frequencies that have occurred in populations due to epidemics.

The Genetics of Asthma, a Complex Disease

Asthma, which can be considered a consequence of an overly sensitive immune system, is a substantial and growing health problem. As of the year 2000 it was the eighth-most prevalent chronic disease in the United States and affected about fifteen million Americans. That's an increase of more than fifty percent between 1982 and 1996. While this dramatic increase underscores a clear environmental component asthma is also a genetic disease. The likelihood for getting asthma varies widely and has been known to run in families. Identical (monozygous) twins have a higher concordance of their asthma susceptibility than do fraternal (dizygous) twins.

The genetics of asthma, like the genetics of most prevalent diseases, is complex. There is no single gene for asthma, coronary heart disease, or most forms of cancer. Moreover, the severity of asthma-related symptoms follows a continuum. During the 1990s geneticists have been increasingly able to map complex, continuous conditions to regions of the genome. About a dozen different regions of the genome have been identified for having effects on asthma susceptibility. Interestingly, asthma propensity maps to different genetic regions depending on which ethnic group(s) are studied. As summarized by Matt Ridley, "the gene that most defined susceptibility to asthma in blacks was not the same that most defined susceptibility in whites, which was different again from the gene that most defined susceptibility in Hispanics."[1]

Why could this be? Michael Wade presents a plausible explanation for this failure to replicate the results in different populations: that the genetic background is different across the different populations.[5] The different populations could have somewhat different allele frequencies of genes that act as modifiers of the genes that have a large effect on asthma propensity. This would be consistent with we know about genetic variation in human populations: differences among populations are usually ones of frequency, not of kind. Because of different frequencies of modifier alleles across the populations, a particular gene may explain more of the variation in asthma sensitivity. Determining whether this is the explanation for the different results obtained for asthma susceptibility will require first isolating the modifier genes and then testing whether their frequencies vary in different populations.

Our History, Our Future

The common ancestor that we shared with chimpanzees about six million years ago was much more like modern chimps than us. In our lineage, the hominids, so many changes occurred: bipedalism, substantially larger brains, tool use, language, and so on. The genetic bases of these important transitional changes remain murky at best. What genetics has shown us is that we are one species, somewhat lacking in genetic variation, and having only slight differences among different populations. Genetic studies have also shown that disease and other factors continue to substantially affect our evolutionary trajectory.

References

1) Ridley, M. *Genome*: *The autobiography of a species in 23 chapters.* Perennial.

2) Lewontin, R. C., S. Rose, and L. J. Kamin. 1984. *Not in our genes: Biology, ideology and human nature.*

3) Wills, C. 1995. When did Eve live? An evolutionary detective story. *Evolution* 49:593–607.

4) Ovchinnikov, I. V., A. Gotherstrom, G. P. Romanova, V. M. Kharitonov, K. Liden, and W. Goodwin. 2000. Molecular analysis of Neanderthal DNA from the northern Caucasus. *Nature* 404:490–93.

5) Wade, M. J. 2001. Epistasis, complex traits and mapping genes. *Genetica* 112/113:59–69.

Further Reading

Books

Freeman, S., and J. C. Herron. 2001. *Evolutionary analysis.* 2d ed. Upper Sadle River, NJ: Prentice Hall.
An excellent inquiry-based college-level textbook on evolution.

Ridley, M. 2000. *Genome: The autobiography of our species in 23 chapters.* New York: HarperCollins.
A series of essays (one for each chromosome in the human genome) that discuss various aspects of human genetics and evolution.

Willis, C. 1998. *Children of Promethus: The accelerating pace of human evolution.*
In this semi-popular book, Willis discusses the ways in which humans are still evolving.

Glossary

Balancing selection. Selection actively maintains more than one variant of a gene in a population.

Chemokine. A chemical signal that attracts white blood cells to infected parts of the body.

Chemokine receptor. Protein associated with the membranes of white blood cells that chemokines can attach to.

Clade. An organizational term used in cladistics to describe a group of related organisms being compared.

Gene tree. A representation of the evolutionary history of a particular gene or DNA sequence.

Hominids. All members of the lineage that includes Homo sapiens and all extinct species since it split from the common ancestor of humans and apes.

Mitochondrial Eve. The woman who possessed the most recent common ancestor of all mitochondrial DNA variants currently in the human population.

Molecular clock. The hypothesis that, within lineages, DNA sequences of a particular gene will evolve in a roughly clock-like manner; that is, approximately as a linear function of time.

Multiregional hypothesis. The hypothesis that gene flow between different regional populations of archaic *Homo sapiens* allowed them to all evolved together into modern Homo sapiens; contrasted with the out of Africa hypothesis.

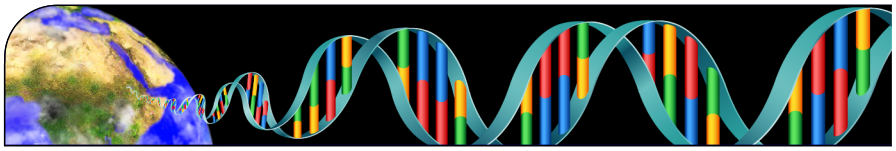
"Out of Africa" (Replacement hypothesis). The hypothesis that postulates that modern *Homo sapiens* spread out of Africa into Europe and Asia and replaced archaic Homo sapiens living in those regions; contrasted with the out of africa hypothesis.

Polymorphism. The presence of two or more variants of a genetic trait in a population.

Species tree. A representation of the evolutionary relationships of different species.

Sister taxa. The most closely related groups of organisms in a phylogeny.

Taxa. Groups or representatives of related organisms that are being compared; they can vary in hierarchical level (such as genus, family, order, and so on).



REDISCOVERING BIOLOGY

Molecular to Global Perspectives

Neurobiology

"The human nervous system is probably the most intricately organized aggregate of matter on Earth. A single cubic centimeter of the human brain may contain well over 50 million nerve cells, each of which may communicate with thousands of other neurons in information-processing networks that make the most elaborate computer look primitive. These neural pathways control our every perception and movement and enable us to learn, think, and be conscious of ourselves and our surroundings."
Campbell and Reece[1]

The most striking differences between humans and other animals are in the size and the complexity of our brains. With our big brains we have acquired a rich culture, which far exceeds that of any other species in scope and complexity. We have developed science to understand how and why an immensity of things and processes work, including those of our own brain. At the start of the twenty-first century neuroscientists are increasingly able to explain the functions of brain in molecular terms.

To understand how the brain works we first must consider what the brain does. This can be broken down into three basic functions: (1) take in sensory information, (2) process information between neurons, and (3) make outputs. The neurons that take in information from the environment are called sensory neurons. These are specialized to respond to a particular stimulus, such as light, heat, chemicals, or vibration — anything you might encounter from outside, or even inside, the body. The processing within the brain can range from a knee-jerk reaction — which takes place entirely in the spinal cord — to the strategy adopted by a master chess player. In humans, we usually call this "thinking." The output is most often a body movement, which results from the action of motor neurons. The brain is the link between the outside world and behavior, and is thus crucial for survival. These three basic functions are shared by organisms from humans down to invertebrates like *Caenorhabditis elegans,* a nematode that doesn't even have a true "brain" but a collection of about three hundred neurons. (See the *Genes and Development* unit.)

But how does the individual neuron work to carry out these tasks? Neurons' unique systems capabilities arise from their cellular ability to communicate with one another very rapidly, using both electrical and chemical communication. Keep in mind, however, that the neuron is not the only type of cell in the brain. The neuron may be the star of the show but there are other supporting players. Indeed, neurons

constitute only a small fraction of cells in the brain. For every neuron there are about ten to fifty supporting cells, called glial cells, in the brain. The word "glial" means glue, and these cells are the "glue" of the nervous system. They perform many vital tasks, including removing dead neurons and debris, releasing critical growth factors to neurons, and acting as insulating material for the neurons.

The incredibly complex ways in which brains function exemplify the importance of cell-cell interactions. Below we discuss the chemical and electrical means by which neurons communicate, and describe how various therapeutic and recreational drugs alter these processes at the molecular level. We then turn to the molecular nature of memory and learning. Finally, we describe recent studies that demonstrate that new neurons are being produced continuously in us.

The Neuron as a Battery

The neuron is an extraordinarily specialized cell. Most neurons are referred to as "bipolar"; they have a cell body and many small extensions, called dendrites, at one end which receive information. (**Fig. 1**) At the other end is its most striking feature: a long axon that ends in "synaptic terminals," which send signals to the dendrites of an adjacent neuron. The longest axon in the human body, the one that goes from the base of the spinal cord to the big toe, is about one meter long. Early studies on the physiology of neurons examined those from the giant axon of the squid, which is so big that it is visible with the naked eye. Note that the neuron, in addition to its specialized functions, carries out nearly all of the functions of a normal cell, except for division.

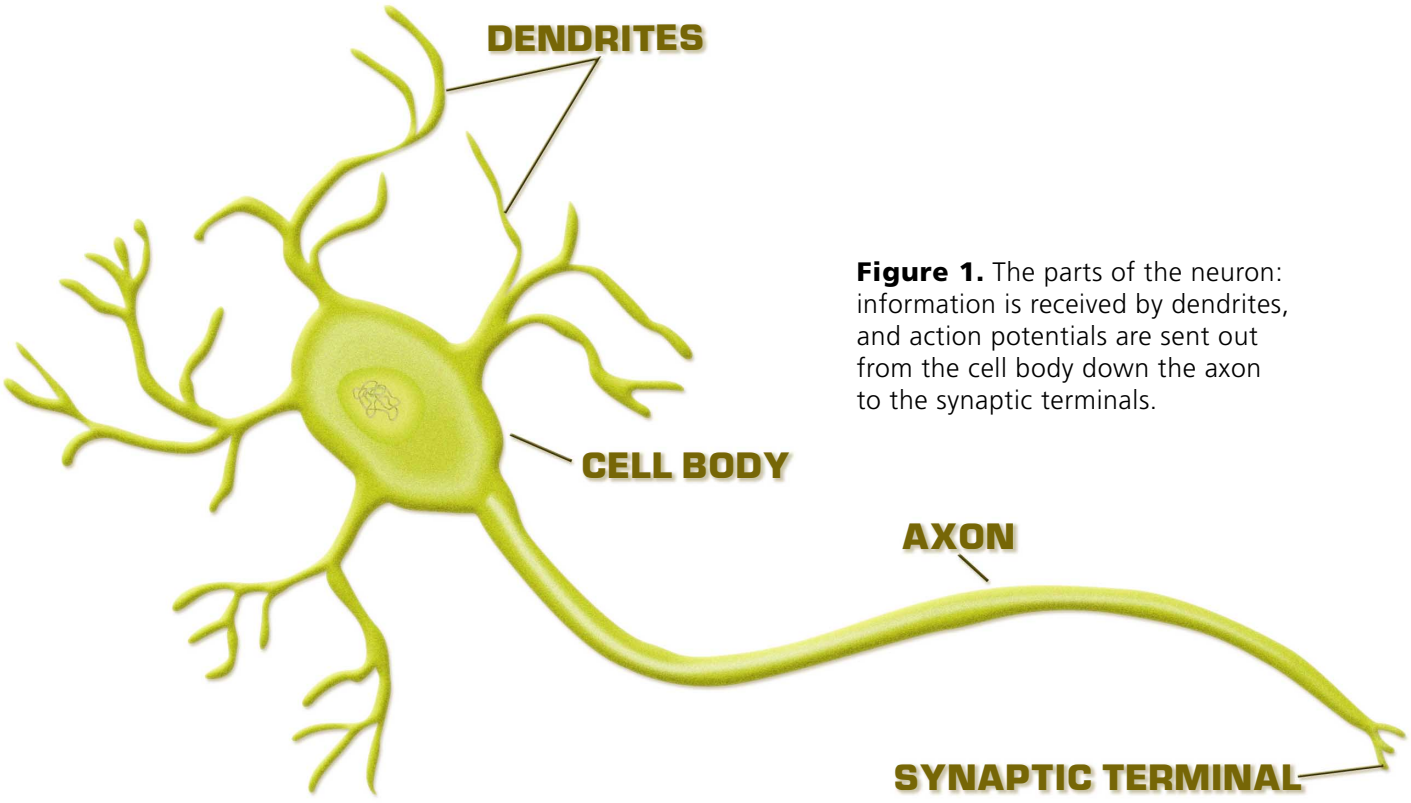


Photo-illustration — Bergmann Graphics

Figure 1. The parts of the neuron: information is received by dendrites, and action potentials are sent out from the cell body down the axon to the synaptic terminals.

The neuron is an electric battery and works by changes in its voltage. Compared with its surroundings, the inside of a "resting neuron" has a lower concentration of sodium ions and a higher concentration of potassium ions. Because of this imbalance of positively charged ions across the membrane, the inside of the resting neuron is negative relative to the outside. This difference in voltage is called the membrane potential. A typical **membrane potential** for a neuron at rest, the **resting potential**, is -0.07 volts, or -70 mV. Although this is a rather modest voltage (about five percent of that of an AA battery), consider that this voltage occurs across a miniscule length — that of the cell membrane. If this were an electric field, the charge separation would be about 100,000 volts per centimeter.

Note that the term "resting neuron" refers only to its electrical state. The cell is really not at rest because, in addition to carrying out all of the normal functions of the cell, the neuron has to maintain this ionic imbalance. This is achieved by the sodium-potassium pump, which actively transports potassium in and sodium out. The pump maintains a negative voltage because it actually pumps three sodium ions out for every two potassium ions it pumps in. The membrane potential of a neuron at any given time is the product of many variables, including the imbalance of ions across the membrane and the membrane's permeability to each ion. In addition to sodium and potassium, chloride is an important ion in "setting" a neuron's rest potential because negatively charged chloride ions can pass through open "leak channels" at rest. Another ion crucial for neural communication is calcium, which acts as a powerful intracellular signaling molecule once it enters through its ion channels.

Voltage-Gated Channels

The neuron, like all cells, possesses a cell membrane that is mostly lipid. Ions like sodium and potassium cannot cross the lipid membrane on their own. In all cells transport of ions, as well as some small molecules, is carried out by channels, which are very tiny openings in the membrane formed by protein pores. These channels are often gated — that is, opened or closed — depending on the conditions of the cell. When open, the ions can enter and pass through channels by diffusion. Ions will always travel down their electrochemical gradient. For example, sodium is much more plentiful outside the cell than inside. It is also positively charged, while the inside of the cell is typically negatively charged relative to outside. Thus, both the chemical and electrical components of the gradient will drive sodium ions into the cell when sodium channels open. **Voltage-gated channels** are those in which the membrane potential of the cell determines whether they are opened or closed. Other channels can be opened or closed by various chemicals, such as neurotransmitters.

Channel proteins that span the cell membrane form the ion channels. To determine the structure of proteins, scientists have often used **X-ray crystallography**. (See the *Proteins and Proteomics* unit.) In 2003 Roderick MacKinnon and his colleagues used this technique to examine the structure of a voltage-gated potassium channel from a unicellular archaea. Previous studies have shown that ion channels have a central ion-conduction pore. Like all proteins, ion channel proteins are made up of amino acids, some of which are charged. When voltage changes occur, these charged components of the protein

make very small movements. This can result in more dramatic conformational changes, causing the channels to open and close. MacKinnon's group found that "voltage-sensor paddles" surround this pore. It appears that with voltage changes in the membrane, these paddles will move and thus permit potassium ions across the membrane.[2] Further study of the structure of the different classes of ion channels from other species will help elucidate the mechanisms by which they allow ion transport.

The Action Potential

What is a nerve impulse? A nerve impulse, or an **action potential**, is a series of electrical responses that occur in the cell. (**Fig. 2**) With the appropriate stimulation, the voltage in the dendrite of the neuron will become somewhat less negative. This change in the membrane potential, called **depolarization**, will cause the voltage-gated sodium channels to open. Sodium ions will rush in, resulting in a rapid change in the charge. At the peak of the action potential, that area of the neuron is about 40 mV positive. As the voltage becomes positive, the sodium channels close, or inactivate, and the voltage-gated potassium channels open. These potassium channels let potassium ions rush out of the cell, causing the voltage to become negative again. The potassium channels remain open until the membrane potential becomes at least as negative as the resting potential. In many cases, the membrane potential becomes even more negative than the resting potential for a brief period; this is called **hyperpolarization**. An action potential typically lasts a few milliseconds.

Figure 2. A cross-section of an axon, with an action potential (AP) moving from left to right. The AP has not yet reached point 4; the membrane there is still at rest. At point 3, positive sodium ions are moving in from the adjacent region, depolarizing the region; the sodium channels are about to open. Point 2 is at the peak of the AP; the sodium channels are open and ions are flowing into the axon. The AP has passed by point 1; the sodium channels are inactivated, and the membrane is hyperpolarized.

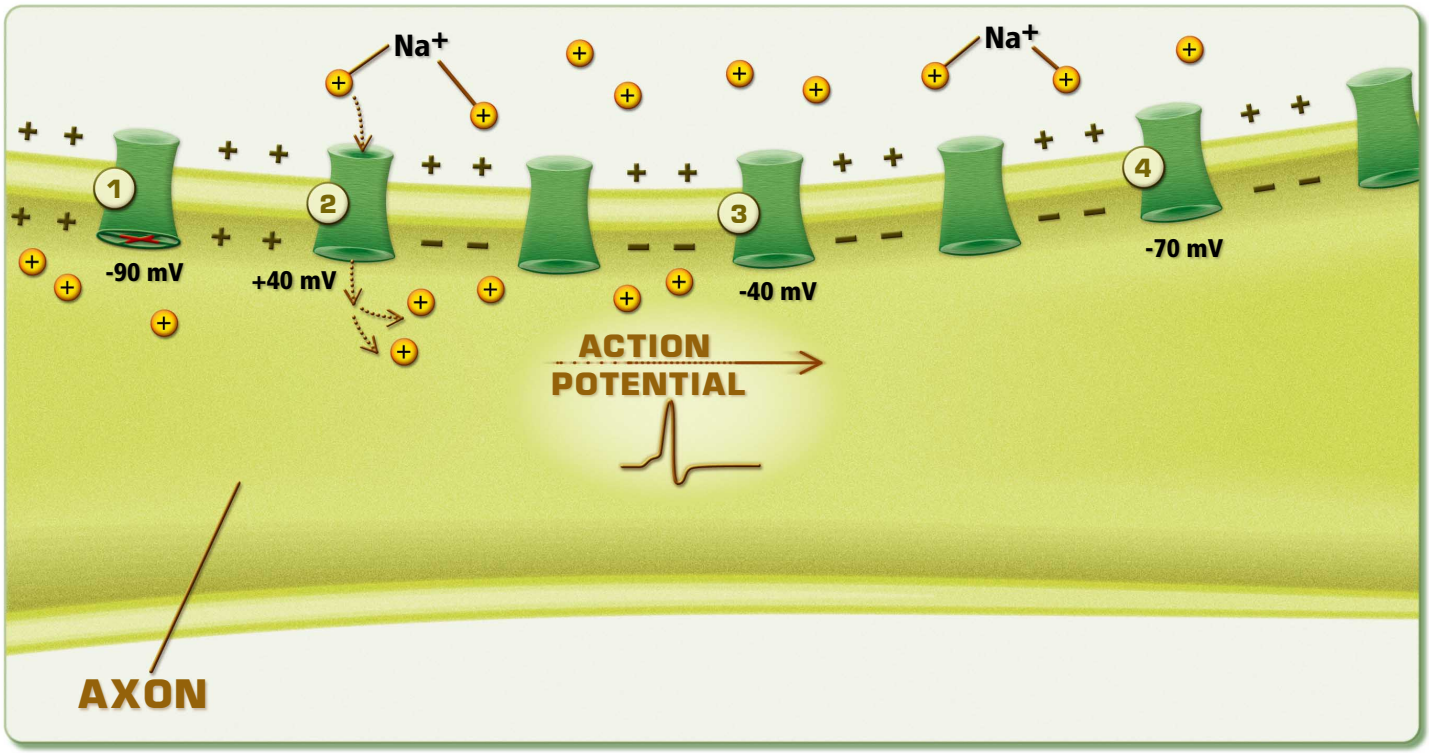


Photo-illustration — Bergmann Graphics

How can this action potential be propagated along the neuron? When the sodium channels are opened, sodium ions rush in; once inside they cause nearby regions of the neuron to become depolarized by moving laterally through the axon. This, in turn, causes the opening of more voltage-gated sodium channels in those regions. Thus, the sodium channel activation moves in a wave-like fashion: the action potential is propagated down the length of the neuron, from its input source at the dendrites, to the cell body, and then down the axon to the synaptic terminals. How does the action potential maintain this directional flow that is key to information processing? The sodium channels have a mechanism that avoids "back propagation" of the action potential, which would result in a confused signal. After opening, the sodium channels become inactivated as the potential becomes more positive, and they cannot open again until they are "reset" by hyperpolarization at the end of an action potential. This brief period of sodium channel inactivation, called a refractory period, prevents bidirectional propagation of the action potential, constraining it to go in only one direction.

Myelin Speeds Up Thought

Most neurons have a fatty outer layer called myelin, which insulates and protects the axons of neurons. In this way, myelin is like the plastic that surrounds electric wires. Myelin is actually made up of two special classes of glial cells, called the oligodendroglia and Schwann cells, which wrap themselves around the axon much like a jellyroll. Between these cells there are small gaps in the myelin sheath called the Nodes of Ranvier. Action potentials are able to jump from one node to the next one down the neuron incredibly rapidly. For this reason, impulses will travel down a myelinated neuron faster than they will across an unmyelinated neuron. In myelinated neurons, action potentials usually travel at over 100 meters per second, which is about half the speed of sound. In about one-hundredth of a second, an action potential can travel from the brain to the base of the spinal cord of an adult. Though seemingly instantaneous, this rate is still on the order of a million times slower than electricity.

Several degenerative diseases are due to the loss of myelin in certain neurons. The loss of muscle coordination that people with multiple sclerosis face is due to the degeneration of the myelin sheath in classes of neurons that are involved in the movement of muscles. The disease is suspected to be an autoimmune disorder — the immune system attacks the myelin sheaths. While MS is usually strikes first in early adulthood, many other diseases that are due to myelin degeneration occur in infancy or early childhood.

Across the Synapse

How is information transferred from one neuron to the next? Neurons communicate at their meeting points, called **synapses**; the small gaps separating the neurons are referred to as the synaptic space. These synapses are not merely gaps but are functional links between the two neurons. Signals are transferred in only one direction across the synapse. The neuron that transmits information when it fires is called the **presynaptic neuron**. The synaptic terminals of the presynaptic neuron are on one side of the synapse; the dendrites of the other neuron, the **postsynaptic neuron**, are on

the other side. Presynaptic and postsynaptic are relative adjectives; a postsynaptic neuron at one synaptic connection can be a presynaptic neuron at another synapse.

Synapses can be either chemical or electrical. An electrical synapse is what is often called a "gap junction," in which the membranes of two neurons are continuous at tiny spots, making the cells electrically contiguous. Gap junctions, which are not unique to neurons, allow for even more rapid communication. No chemical intermediary is involved in an electrical synapse. In the case of chemical synapses, however, chemicals called **neurotransmitters** are released from a presynaptic neuron, and dock with receptor proteins on the postsynaptic neuron. Such binding causes the shape of the protein to change and ion channels to open, much like the voltage-gated channels open in response to membrane potential changes (**Fig. 3**). We will discuss neurotransmitters in more detail below. Neurons are typically separated by about twenty to thirty nanometers in chemical synapses. Electrical synapses are more rapid than chemical ones but chemical synapses are easier to modulate. In vertebrates and many invertebrates, chemical synapses are more common than are electrical ones.

The action of the presynaptic neuron is referred to as an "all or none" response. A neuron can only fire or not fire; there is no "slightly activated" signal from a neuron. Whether or not a neuron will fire an action potential — that is, send a signal down its axon to be received by other neurons — depends on how many inputs it is receiving. It also depends on the nature of each input signal — excitatory or inhibitory — at each synapse. The sort of "net total" result of those signals determines whether the neuron will become excited, or depolarized, enough to fire an action potential and release neurotransmitter from its axon terminals.

Also recall that a signal traveling through the brain often involves many neurons, each making so many connections. This interconnectedness gives rise to the extraordinary complexity of the brain. The activation of a single sensory neuron could quickly lead to the activation or inhibition of thousands of neurons.

Neurotransmitters and Receptors

Neurotransmitters are usually small molecules, such as amino acids (e.g., glutamate and aspartate) and amines (e.g., dopamine, serotonin, and histamine). Some neurotransmitters stimulate neurons to fire, while others inhibit firing. The effect of the neurotransmitter comes about by its binding with receptor proteins on the membrane of the postsynaptic neuron. Each neurotransmitter binds specifically in a lock-and-key mechanism to its type of receptor. Neurons in different pathways will often have different types of receptors in a given family. For example, dopamine binds to dopamine receptors, but there are about a dozen subtly different dopamine receptors. Neurobiologists think that the human nervous system uses at least fifty neurotransmitters, but about ten carry out most neurotransmission. Many of these neurotransmitters are highly conserved in other organisms. Most neurons release only one type of neurotransmitter.

Neurotransmitters are released in a process called **exocytosis**. When the action potential reaches the end of an axon the depolarization causes calcium channels to open. The calcium causes **synaptic vesicles**

Figure 3. Synaptic vesicles fuse with the presynaptic membrane to release neurotransmitter into the synaptic space. Here, they bind with neurotransmitter receptors in the postsynaptic membrane.

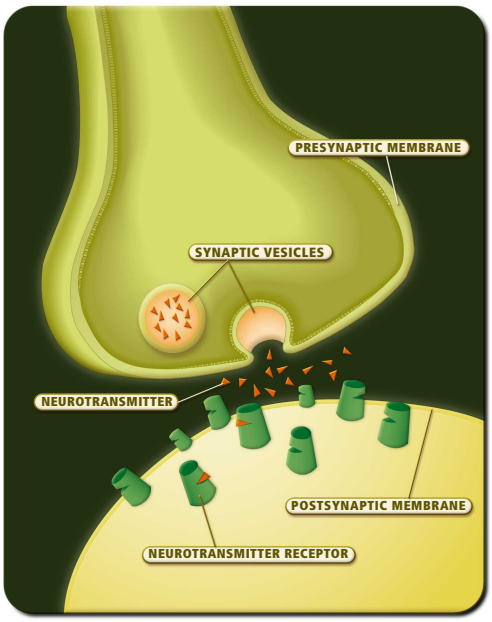


Photo-illustration — Bergmann Graphics

that carry the neurotransmitter to fuse with the cell membrane. This fusion allows the neurotransmitter to be released into the synapse. Although exocytosis occurs in many cell types, neurons use a specialized form in which calcium causes a chain of events that culminates in fusion of the vesicles.

There are two general categories of receptor proteins: **ionotropic** and metabotropic. Activation of ionotropic receptors causes membrane ion channels to open or close. In contrast, activation of metabotropic receptors involves an intracellular biochemical cascade. Such a cascade may end with the opening or closing of ion channels or other intracellular effects.

As long as the neurotransmitter remains in the synapse, it will continue to bind its receptors and stimulate the postsynaptic neuron. At some point the signal is no longer needed. Moreover, continual stimulation can injure some neurons. So, halting the stimulus is just as important as the appropriate starting of the stimulus. How does the neurotransmitter leave the synapse? There are several ways, such as diffusion away from the synapse or breakdown of the neurotransmitter by specific enzymes. Another common mode, called **reuptake**, involves specialized molecules present on the membrane of the presynaptic neuron. These molecules, called **neurotransmitter transporters**, have receptor sites that will bind to the neurotransmitter and actively transport it out of the synapse, back to the presynaptic neuron. That neuron can then reuse the neurotransmitter. The action of several drugs takes place at the reuptake stage.

Neurotransmitters, Psychoactive Drugs, and the Reward Pathway

Drugs that have effects on the central nervous system are known as psychoactive drugs. The mode of actions of both therapeutic drugs (e.g., Ritalin, Prozac, and Paxil) and recreational drugs (e.g., alcohol, cannabis, cocaine, and nicotine) affect the firing of certain neurons by changes in various neurotransmitters or receptors. Not all drugs have specific modes of action; alcohol, for example, has many and varied effects. We will focus, however, on a few examples of those drugs that have specific effects.

Humans and many other animals engage in many activities from which they derive pleasure. Researchers working with various animals have shown that there are regions of the brain, such as the ventral tegmental area, that are more active when animals engage in pleasurable acts. When researchers stimulate these areas experimentally, the animals will perform various tasks in order to receive further stimulation. Hence, the neural pathway comprises those regions has been called the **reward pathway**.

Like many drugs, nicotine from tobacco products acts on the reward pathway. This drug, however, is unusual in that it directly affects the dopamine receptor in the reward pathway's neurons. Unlike the action of most drugs, no intermediary steps are involved: nicotine binds to the receptor and stimulates the postsynaptic neuron. The overstimulation of the postsynaptic cell, however, also has effects at the cellular level. Over time, it leads to a decrease in the number of dopamine receptors being expressed and inserted to the membrane, as well as a change in the shape of the cell. The reduction of receptors is

referred to as desensitization. When the nicotine is removed, because there are fewer receptors on the postsynaptic cell, more dopamine than normal is required for proper stimulation of postsynaptic neuron. Addiction can result because nicotine becomes needed just to maintain the normal stimulation of the postsynaptic cells.

Allelic variation at the dopamine receptor gene appears to affect one's likelihood of becoming addicted to nicotine. Individuals who have the A1 allele have fewer dopamine receptors than those that do not have the allele. These individuals also have more difficulty in quitting smoking and are more likely to exhibit other addictive and compulsive behaviors. The genetic components of many types of addiction are the topic of intensive research — and often heated debate.

Cocaine also works on dopamine and the reward pathway but does so in a different way. Recall that some neurotransmitters are normally taken up by the presynaptic neuron by reuptake receptors, or transporters, in the presynaptic membrane. (**Fig. 4**) The molecular structure of cocaine is such that it can block the binding site for dopamine on its reuptake receptor. Because this cell is now impaired in the reuptake of dopamine, an excess of dopamine builds up in the synapse. This excess leads to overstimulation of the postsynaptic neuron. Because the action is occurring in the reward pathway, overstimulation leads to euphoria. The effects of overstimulation of the postsynaptic cell by cocaine are much the same as those of nicotine: the reduction of the number of receptors leads to desensitization and the possibility of addiction.

Figure 4. Left: Dopamine in the synaptic space binds to dopamine receptors on the postsynaptic cell. Dopamine transporters in the presynaptic membrane take up the dopamine molecules from the synaptic cleft and return them to the presynaptic cell. Right: Cocaine blocks the reuptake of dopamine, leading to molecular changes that contribute to addiction.

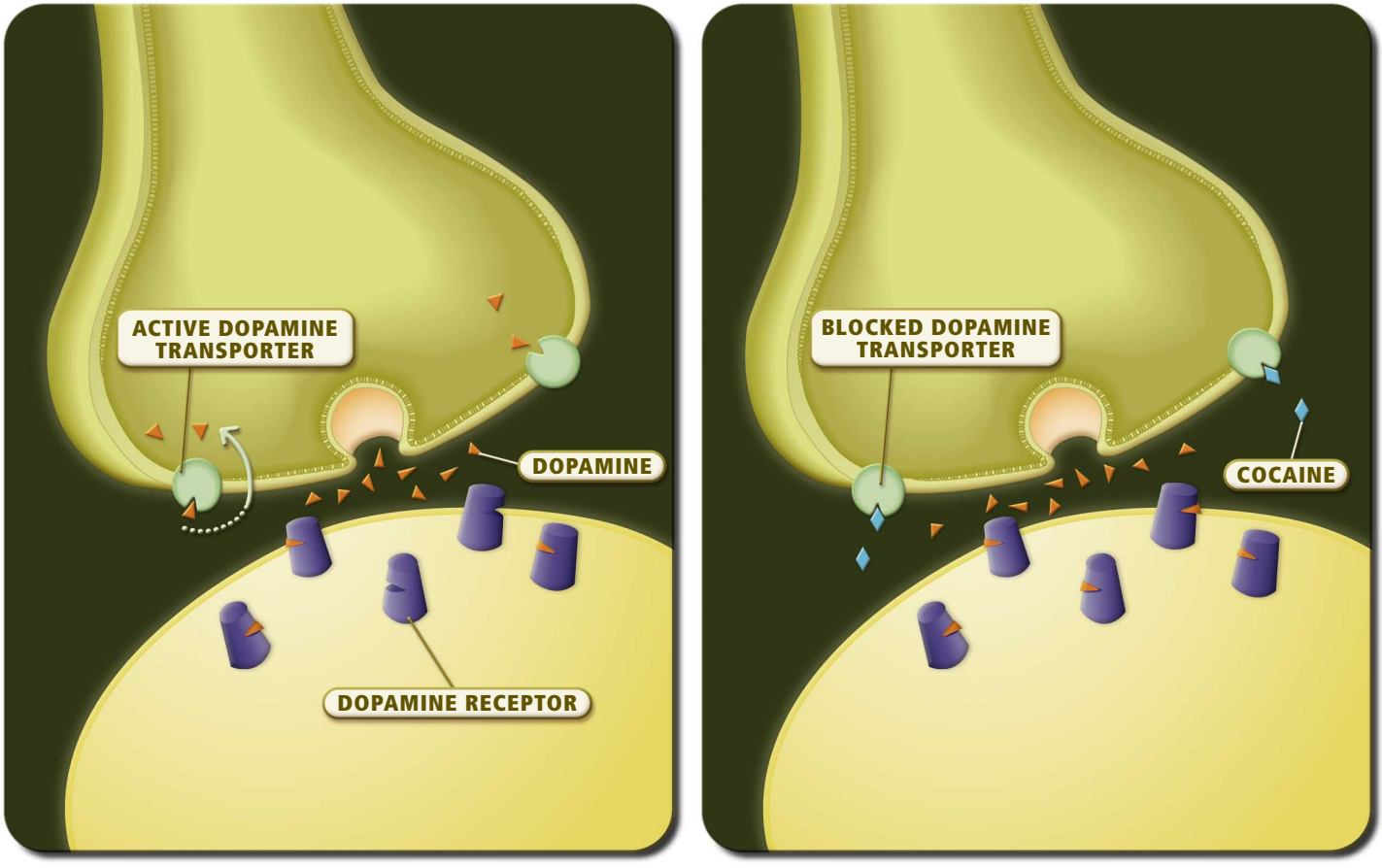


Photo-illustration — Bergmann Graphics

There have been concerns that Ritalin (methylphenidate), used for treatment of attention deficit and hyperactivity disorder (ADHD), is chemically similar to cocaine. Indeed, Ritalin increases dopamine levels by interfering with reuptake. Moreover, Ritalin and cocaine compete for the same receptor site. One crucial difference between these two drugs is that Ritalin acts much more slowly than cocaine. While cocaine's effects on dopamine levels occur within seconds, the response from Ritalin (when administered in pill form) takes about an hour. Some studies suggest that, far from leading to addiction, Ritalin treatment in childhood may be associated with decreased risk of drug and alcohol use later on. Other studies, however, suggest that Ritalin may be a gateway drug: by using it, teens may be more willing to experiment with other drugs. As of 2003 the consequences of Ritalin treatment remain unresolved. (**Fig. 5**)

Figure 5. The chemical structures of dopamine, Ritalin, and cocaine are structurally similar: they all bind at the dopamine transporter, affecting reuptake of dopamine.

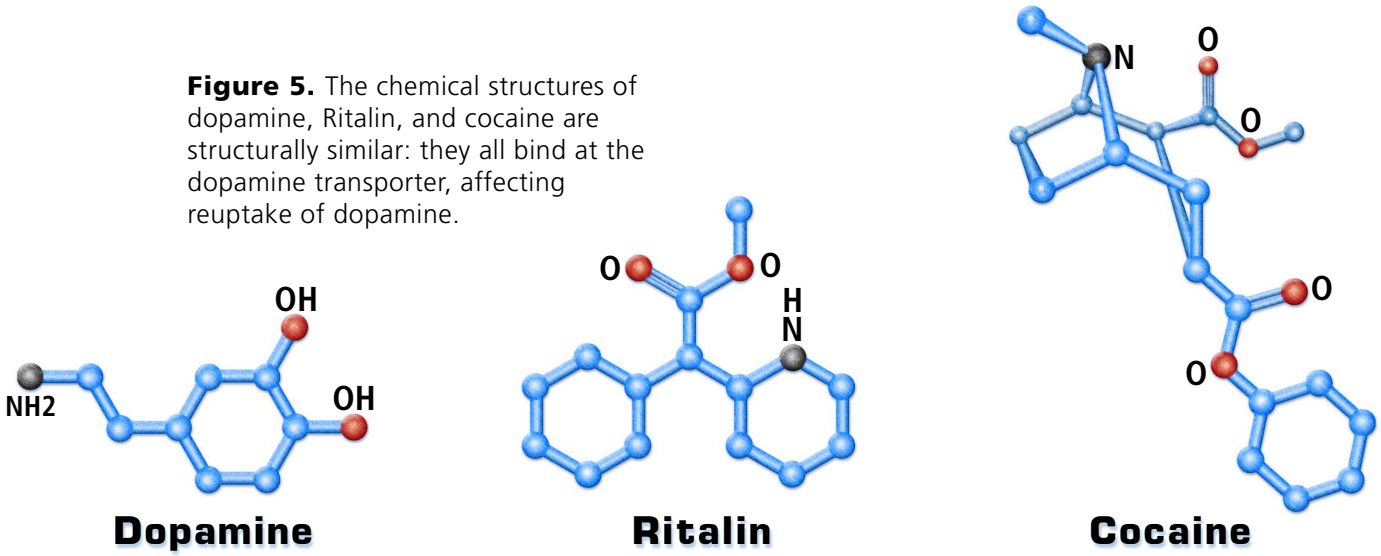


Illustration — Bergmann Graphics

Prozac and Serotonin Reuptake

Soon after it was released to the market in 1988, Prozac (fluoxetine hydrochloride) became the most prescribed drug to treat depression. It and several other antidepressants inhibit the reuptake of serotonin, a neurotransmitter that affects mood, sleep, and appetite. These drugs are called selective serotonin reuptake inhibitors (SSRIs) because, unlike older antidepressants, they have little effect outside of serotonin reuptake. By inhibiting the reuptake of serotonin, Prozac and SSRIs increase the level of serotonin in the synapses. The increased levels of this neurotransmitter generally result in an improved mood. Depressed patients often had lower than normal levels of serotonin.

Cannabis, the Cannabinoid Receptors, and Endocannabinoids

The active ingredient of marijuana, from the cannabis plant, is THC (delta-9-tetrahydrocannabinol). This chemical exerts its effects on the brain by binding to receptors called the cannabinoid receptors. Scientists have identified two cannabinoid receptors (CB1 and CB2), and evidence suggests that there may be others. Although CB1 is found in many regions of the brain, CB2 is present only in certain cells

of the immune system. Because the receptor is present in several brain regions, THC can have manifold effects. For instance, THC may affect memory formation. CB1 is prevalent in the hippocampus, a region of the brain strongly associated with memory. By binding to and activating CB1, THC decreases activity of neurons in the **hippocampus** and interferes with the proper function of that region, which may translate to an interference with memory formation.

The human body does not produce THC, so why would there be receptors that can bind it? During the 1990s researchers discovered that the body makes chemicals, such as anandamide, that can bind to the cannabinoid receptors. The function of these chemicals, called endocannabinoids, and their receptors is still unknown. To investigate the role of the CB1 receptor, scientists have studied mutant mice that lack the receptor. Compared with normal mice, these mice have a decreased appetite, are less active, and have a reduced lifespan; however, the mice have an enhanced memory.

The CB receptors have recently been associated with some beneficial actions, such as pain relief and extinguishing some fear behaviors. THC has even been prescribed as medication in some states for pain relief for various diseases, including glaucoma, AIDS, and cancer.[3]

The Molecular Basis of Learning and Memory

It is clear that an understanding of mechanisms at the level of the synapse explains changes in our behaviors, like movements. But what about longer-term changes associated with learning and memory? Can they be understood in molecular terms, too? Memory, and thus learning, involves molecular changes in the brain. During the last few decades, researchers have started to map the molecular processes involved in memory formation. They have been increasingly able to link the ability to remember with physical changes in the structure of neurons.

One important change that occurs in memory formation is **long-term potentiation (LTP)**. This phenomenon involves the long-term modification of the synaptic communication. Under normal circumstances the rate at which a postsynaptic neuron fires depends on how much stimulation it receives from presynaptic neurons. Once the increased stimulation has stopped, the postsynaptic neuron will return to its normal rate of firing. In LTP, however, the postsynaptic neuron will continue to fire at an elevated rate, even after the increased stimulation has subsided. It seems to become more sensitive — or gives a bigger reaction by firing more action potentials — to a given stimulus. How does this happen?

Glutamate is the neurotransmitter involved in LTP. Glutamate can bind to several different types of ionotropic receptors, including the NMDA- (N-methyl-D-aspartate) and AMPA- (amino-3-hydroxy-5-methyl-4-isoxazolepropionate) type glutamate receptors, each of which opens a specific type of channel within the receptor proteins. Both channels are involved in memory formation. The NMDA channel requires both glutamate and depolarization from another source to open. Why? The molecular mechanism is as follows. Normally, at negative potentials, positively-charged magnesium ions plug the pore of the NMDA channel. While glutamate may "open" the pore, the ions cannot travel

through the channel due to the magnesium block. When the membrane is depolarized, however, the inside of the cell becomes more positive, and the magnesium ions are no longer driven into the channel. Thus, the block is relieved, allowing sodium and calcium ions to flow in.

So, this mechanism allows the NMDA-type glutamate receptor to act as a "coincidence detector." When the neuron receives input from only one source — another neuron — glutamate binds to and opens both NMDA- and AMPA-type receptors. (**Fig. 6**) Because the neurotransmitter arrives at a resting, negatively charged, postsynaptic membrane, magnesium ions prevent flow through NMDA channels. When, however, stimulation of a neuron occurs simultaneously from more than one source — say several other neurons — some glutamate will bind NMDA receptors in parts of the neuron that are already depolarized, or less negatively charged.

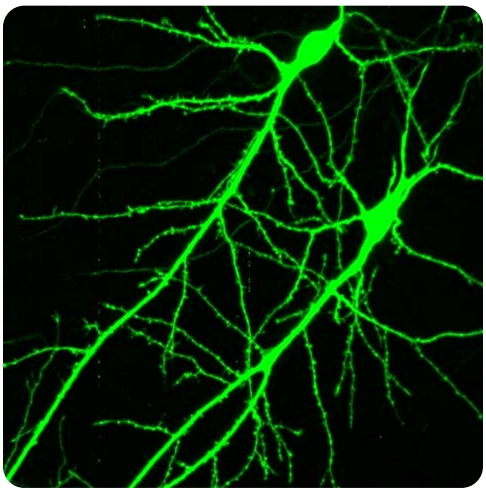
Where does this voltage change come from? Recall that once an action potential has started, it spreads from its source throughout the entire membrane of the neuron in a wave-like fashion; thus, other dendrites may be "pre-depolarized" before glutamate binds. In this cas, the block by magnesium is relieved and the NMDA channel also passes ions. While AMPA channels can pass only sodium ions in, NMDA channels also pass calcium. This calcium permeability gives the NMDA channel its ability to trigger LTP.

Now that we have examined the requirements for LTP, what is the effect? When calcium ions rush in, they set off an intracellular signaling cascade that can involve dozens of molecules. Speculation about the identity and functions of these molecules has been the subject of intense scientific inquiry since the early 1990s — it was perhaps the most studied aspect of neuroscience during that "decade of the brain."

So how could this intricate electrical mechanism act to form new memories? LTP, like learning, is not just dependent on increased stimulation from one particular neuron but on a repeated stimulus from several sources. It is thought that when a particular stimulus is repeatedly presented, so is a particular circuit of neurons. With repetition the activation of that circuit results in learning. Recall that the brain is intricately complicated. Rather than a one-to-one line of stimulating neurons, it involves a very complex web of interacting neurons. But it is the molecular changes occurring between these neurons that appear to have global effects. LTP can lead to strengthened synapses in a variety of ways. One such way, as discussed in the video, is by the **phosphorylation** of glutamate receptor channels, which is accomplished by a calcium-triggered signaling cascade. This results in those channels passing more ions with subsequent stimulation, strengthening the signal to and from the neuron.

But more permanent changes — long-term memory — require the synthesis of new proteins. In a variety of organisms, including flies (*Drosophila*) and humans, one enzyme, CREB (cyclic-AMP response element binding protein), seems to be involved in the steps that facilitate this new protein expression. When calcium flows in through NMDA channels, one of the molecules it activates is CREB. In turn, activated CREB acts as a **transcription factor** (see the *Genes and Development* unit) that activates the expression of other genes. This

Figure 6. Two hippocampal neurons, labeled with green fluorescent protein, viewed with confocal microscopy. Such neurons release and sense glutamate, and engage in long-term potentiation (LTP). Note the synaptic connections between the lateral processes of the two neurons.



Courtesy of Rick Huganir, PhD.

gene expression can lead to the production of more ion channel receptors, as well as structural proteins like actin, which cement the synaptic connection between two repeatedly communicating neurons.

Mutant mice lacking the NMDA receptors show severe deficiencies in memory tasks. On the other hand, researchers have genetically engineered (see the GMOS unit) mice that have more of the NMDA receptors. These mice, dubbed "smart mice" by the popular press, are substantially better at several memory tasks than are normal mice.

Memory and the Hippocampus

Psychologists have long argued that there are many different types of memory. These can be classified by many criteria, based on decades of experimental research and the different memory defects seen in people who have suffered brain damage. Scientists have agreed that memory can be viewed in temporal terms; that is, there is a short-term memory, with a limited capacity for about a dozen items, and a long-term memory, to which these items are presumably transferred for "storage." Short-term memory seems to be much more vulnerable to loss due to trauma than does long-term memory: people may even lose the ability to form new memories, while their ability to remember their entire lives before an accident remains intact. This memory defect is exemplified in the movie *Memento* (2000), in which a widower avenges his wife's murder — during which he suffered brain damage — over and over again. Such individuals with this condition of "anterograde amnesia" usually have severe damage to their hippocampus. As Kempermann points out, the hippocampus is not the equivalent of the brain's hard drive but rather a gateway, "a structure, through which all information must pass, before it can be memorized."[4]

It is widely agreed that while the hippocampus is undeniably important for memory, the "recording" of information into long-term memory involves plasticity, or physical changes, in multiple regions throughout the entire nervous system. Another interesting distinction that scientists have made in types of memory is between declarative memory, which allows you to remember facts and is extremely complex, and reflexive memory, which usually consists of learning by repetition and often involves motor learning. While declarative memory can be reported, reflexive memory is exhibited by performance of a task and cannot be expressed verbally. It is now thought that the two types of memory may involve two entirely different neuronal circuits.

The hippocampus plays a major role in spatial learning and memory in a number of animals. Research with black-capped chickadees and other species of birds has shown that when the hippocampus is removed, the birds still store food but cannot recall where they stored it. Moreover, bird species that rely heavily on stored food as a winter resource in general have larger hippocampi than those species that don't.

Studies of cab drivers in London have provided fascinating information about the role that the hippocampus plays in spatial memory. London cab drivers are known for their navigational skills and knowledge of the streets of London. To learn how to navigate the streets of the city, would-be cab drivers undergo "the Knowledge," a rigorous training that can take two years to complete. Recent studies using magnetic resonance imaging (MRI) demonstrate that the hippocampi of the

London cab drivers are somewhat different. Specifically, the posterior region is significantly larger and the anterior region is significantly smaller in the cabbies when compared with control subjects. Other studies have found that the posterior region is active during tasks involving spatial memory. It is possible that the cabbies come disproportionately from those individuals with excellent spatial memories and corresponding larger posterior regions of the hippocampus. There is further evidence, however, that suggests that the memory work of the cabbies has altered their hippocampi. Those cab drivers that have been working the longest tend to have larger posterior hippocampi than more recently hired cabbies. Furthermore, other imaging studies show that the right hippocampus is activated in the cab drivers when they are asked to remember complex travel routes but not when they are asked to provide information about famous landmarks.[5]

Neuronal Stem Cells

What neuronal processes have led to the changes in the hippocampi of London taxi drivers? Perhaps this is achieved by neurons migrating from one region to the posterior hippocampus? Another intriguing possibility is that the changes are the result of new neurons going to the region.

New neurons? Don't we have our complete store of neurons by early childhood? That previous dominant paradigm had been found incorrect. In the past two decades, researchers have shown that neurons are continually produced in a variety of animals, including humans. It isn't that neurons divide. They don't. Instead, the brain maintains a reservoir of stem cells that are capable of generating new neurons (neurogenesis). One area of the brain where stem cells have been found is the hippocampus.

The discovery of stem cells and neurogenesis began with basic research with songbirds. During each breeding season male songbirds need to recall their mating song. Starting in the 1980s researchers noted that the number of neurons in certain areas of the brain (especially the hippocampus) would increase in male birds around the start of the breeding season. The number of neurons in these areas would decrease after the mating season. This striking evidence led other researchers to look for neurogenesis in the brains of mammals. Studies on rats found substantial neurogenesis. In one part of the hippocampus alone nearly 10,000 new neurons are generated each day in adult rats. Starting in the 1990s Elizabeth Gould of Princeton University found that the adult brains of several species of monkeys also undergo considerable neurogenesis.

Following these animal studies researchers examined whether humans have the capacity for neurogenesis. They studied postmortem brain tissue from humans, using various stains to determine whether new neurons were being generated from dividing progenitor cells. They were able to find such new neurons in the hippocampus, showing that neurogenesis proceeds throughout life in at least some regions of the human brain.

Engaging in mental and physical activity is one important way elderly people can maintain their mental acuity. This aspect of conventional wisdom has been vindicated by medical research. Mental and physical activity reduces the risk of neurodegenerative disorders and improves

the prognosis of stroke patients. Yet, we know little about the molecular mechanisms behind this effect. Studies in mice of neurogenesis in the hippocampus, however, point to one possible reason for why activity keeps the mind sharp. Mice who were exposed to an enriched environment for the second half of their lives showed a dramatic increase in neurogenesis in the hippocampus as compared with control subjects. The hippocampi from the mice that received the enriched treatment also appeared like those of younger animals. These results strongly suggest that activity maintains the proper function of the brain by increasing neurogenesis in the hippocampus.

Elizabeth Gould and other researchers studying neurogenesis think that the new neurons generated in the hippocampus are involved in modulation of the stress response as well as learning. There are some complications, however. Learning enhances neurogenesis but only under certain conditions. Moreover, experimental blockage of neurogenesis interferes with some types of learning but not others.

Our understanding of neurogenesis remains far from complete. Yet, tremendous progress has been made during the last two decades and further progress is expected. In addition to what these studies tell us about how the brain works, they may also pave the way toward treatment of degenerative diseases like Alzheimer's and Parkinson's as well as brain trauma.

References

1) Campbell, N. A., and J. B. Reece. 2002. Biology. 6th ed., 1002 San Francisco: Addison-Wesley Longman, Inc.

2) Jiang, Y., A. Lee, Y. Chen, V. Ruta, M. Cadene, B. T. Chait, and R, MacKinnon. 2003. X-ray structure of a voltage-dependent K+ channel. *Nature*. 423:33–41.

3) Sullivan, J. M. 2002. Cannabinoid receptors. *Curr. Biol*. 12:R681

4) Kempermann, G. 2002. Why new neurons? Possible functions for adult hippocampal neurogenesis. *J. Neuroscience*. 22:635–38.

5) Maguire, E., R. Frackowiak, and C. Firth. 1997. Recalling routes around London: Activation of the right hippocampus in taxi drivers. *J. Neuroscience*. 17:7103–10.

Further Reading

Books

Calvin, W. H., and G. A. Ojemann. 1995. *Conversations with Neil's brain: The neural nature of thought and language*. Perseus Publishing.
Building from case examples, a neurobiologist and a neurosurgeon describe the workings of the brain.

Drickamer, L. C., S. H. Vessy, and E. M. Jakob. 2002. *Animal behavior: Mechanisms, ecology, and evolution*. 5th ed. McGraw-Hill.
A university-level textbook on animal behavior that has an excellent section on the neurobiology of behavior.

Timmons, C. R., and L. W. Hamilton. *Drugs, brains & behavior*. www.rci.rutgers.edu/~lwh/drugs/.
A short e-book detailing the neuropharmalogical effects of drugs.

Article

Sullivan, J. M. 2002. Cannabinoid receptors. *Curr. Biol*. 12:R681.
A short guide to recent research on cannabinoids and their receptors.

Glossary

Action potential. The nerve impulse, or "firing," of a neuron. A traveling wave of depolarized voltage that is propagated along a neuron. Results in the release of neurotransmitter and the movement of information to another neuron.

Depolarization. The state in which the inside of a neuron becomes more positive in voltage than it is at rest.

Hippocampus. A region of the brain associated with memory formation.

Hyperpolarization. A state in which the membrane potential is more negative than is the resting potential; occurs transiently at the end of an action potential.

Ionotropic receptors. Receptors for which neurotransmitter binding results directly in an ion channel opening or closing.

Long-term potentiation. An enduring increase in the strength of the connection between two neurons, which results from repeated stimulation of a given input pathway.

Membrane potential. The difference in voltage between the inside and the outside of a neuron; the outside is always zero.

Neurogenesis. The formation of new neurons from precursor stem cells.

Neurotransmitter. A molecule that travels across the synapse and binds to its receptor on the postsynaptic neuron, influencing its probability of firing.

Phosphorylation. The addition of a phosphate group to a molecule, such as a protein.

Postsynaptic neuron. At a given synapse, the postsynaptic neuron is the receiving neuron at its dendritic end.

Presynaptic neuron. At a given synapse, the presynaptic neuron is the transmitting neuron, its axonal synaptic terminal forms the synapse.

Resting potential. The resting membrane potential of a neuron; it is about -70 mV.

Reuptake. The recapture of neurotransmitters from the synapse back into the presynaptic neuron; accomplished by transporters.

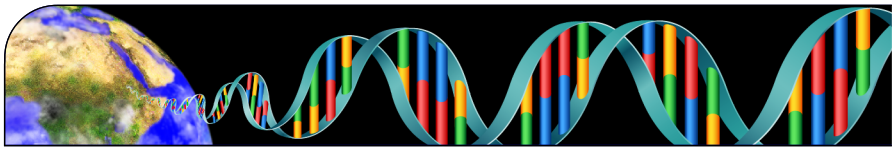
Reward pathway. A pathway in the brain that is stimulated when an animal is engaged in pleasurable activities.

Synapse. A functional connection between two neurons where information can be exchanged in the form of electrical or chemical energy.

Transcription factor. A protein that influences transcription of another gene by binding to DNA.

Voltage-gated channels. Ion channels in the cell membrane that open or close in response to changes in the membrane voltage.

X-ray crystallography. A method for determining the structure of a molecule, such as a protein, based on the diffraction pattern resulting from focused X-ray radiation onto pure crystals of the molecule.



REDISCOVERING BIOLOGY

Molecular to Global Perspectives

Sex and Gender

"I think humans like things to be ordered, and they get bothered about gray areas and when things become less clear-cut. But these days I don't think so much in black and white about male and female. Now I think of it all as being on a spectrum." Dr. Andrew Sinclair[1]

Introduction

Max Beck was born an **intersexual**, someone with ambiguous genitals. Like most babies without a normal penis, he was "assigned" the sex of female and underwent plastic surgery to "fix" his genitals. He was named Judy and grew up as a girl, a self-described tomboy. In his teens, more surgery and female hormone injections turned him into a woman — a woman with no sense of gender identity. As a young adult he had sexual relationships with males and females, first accepting himself as a lesbian, then marrying a man. After divorcing his husband, he once again became a lesbian with a partner. Finally he received his medical records, which revealed that he was an intersexual and had both an X and a Y chromosome. Over a period of two years he decided that he could no longer live as a female. He reassigned himself as a male, married his female partner, and became the father of a child, conceived by his wife using donor sperm. Despite his sex assignment as a female at birth, Max was never able to accept his gender as female.

In contrast, Jan Morris was born James Morris, an apparently normal male. A successful journalist, author, and mountain climber, she married and had five children before she decided in her 30s to change her sex to female and her name to Jan. Jan Morris wrote of her sex change in the book, *Conundrum*, explaining that she had always known that she was a woman, wrongly born into the body of a man. She has continued to be a successful writer and lives harmoniously with her former wife.

The use of pronouns above may strike some as strange. Which pronoun should be used in the case of transgendered individuals? This question highlights the difficulties our language and culture have in confronting issues of sex and gender. We have used the pronoun of the individual's final choice of gender.

What is the difference between sex and gender? Max Beck was born a male (albeit with ambiguous genitals), and efforts to change him into a girl by surgery and hormones failed to change his gender, his sense of identity. In contrast, Jan Morris believes that she was born a woman in a man's body. Transsexuals like Jan Morris explain that they must

change their physical sex because there is no way to change their gender, which comes from the brain. While there is no general agreement on terminology, the Merriam-Webster dictionary defines gender as "the behavioral, cultural, or psychological traits typically associated with one sex," and defines sex as "either of the two major forms of individuals that occur in many species and that are distinguished respectively as female or male." This suggests that sex is biological while gender is subjective.

Sex and the Y Chromsome

Except for the sex chromosomes (X and Y), all humans have the same set of chromosomes. The karyotype of a human male is 46XY (46 chromosomes, including one X and one Y), and that of a female is 46XX (46 chromosomes, including two X chromosomes.) In mammals as a whole, the presence or absence of the Y chromosome usually determines sex. Individuals with an X chromosome but no Y (45XO) are female (Turner's syndrome); individuals with two X chromosomes and a Y chromosome (47XXY) are male (Kleinfelter's syndrome).

The Y chromosome is considerably smaller than the X chromosome and has a much lower density of genes. In fact, the Y has often been called a "genetic junkyard." But there are a few rubies among the rubbish of that genetic junkyard: the Y chromosome contains the genes are essential for male fertility and other male characteristics.

Why does the Y chromosome have so few functional genes? Evolutionary biologists are still debating the details but they agree that the lack of recombination explains the paucity of functional genes on the Y. Unlike the twenty-two pairs of autosomes, there is no recombination between the X and most of the Y chromosome. Genes on the part of the Y chromosome that does not recombine will be passed from father to son, down a paternal lineage, and will never be present in females. The lack of recombination weakens the effectiveness of natural selection to weed out bad variants and select for good ones. Over many millions of years mutations and random genetic drift erode the Y chromosome, turning it into a genetic junkyard. In contrast, genes on the X are present in both males and females; X chromosomes, like autosomes, recombine in production of female gametes.

About five percent of the Y chromosome does recombine with the X. This region, at the tips of the chromosomes, is called the pseudoautosomal region because in it the X and Y chromosomes behave as autosomes (**Fig. 1**). The pseudoautosomal region is more gene-rich than the rest of the Y chromosome. Several of the genes on the pseudoautosomal region of the Y have counterparts on X, reflecting a common evolutionary ancestor. The genes required for male fertility are found in the non-recombining regions of the Y, and are not present on X.

Researchers in David Page's lab have shown that one-quarter of the Y chromosome consists of eight families of nearly identical nucleotide sequences, and includes duplicate copies of important genes. Because these regions are arranged in palindrome fashion, they provide a mechanism for a kind of internal recombination between the similar genes on the same chromosome. This process, called gene conversion, aids in the detection and repair of gene mutations in this part of the Y chromosome.[2]

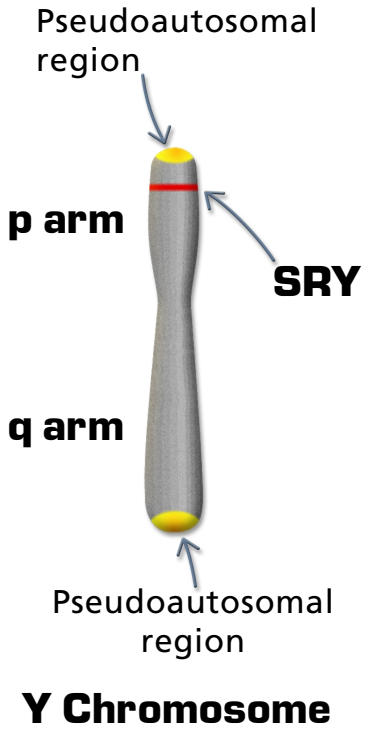


Illustration — Bergmann Graphics

Figure 1. The Y chromosome is very small compared to the X chromosome. The pseudoautosomal regions at the tips contain the genetic material on the Y that shows similarity to the X chromosome. The SRY gene is located on the p arm of the Y.

Paternal Inheritance

The lack of recombination means that the entire non-recombining portion of the Y is passed intact from father to son. A male shares the same Y chromosome with his father, paternal grandfather, paternal great-grandfather, and so on. (**Fig. 2**). Researchers can establish paternal genetic relationships by comparing small differences (polymorphisms) between modern Y chromosomes. The identification of genetic markers such as **single nucleotide polymorphisms (SNPs)** and indels (insertions and deletions) in the non-recombining regions of the Y provides a tool to study population structure and history, genealogy, and human evolution. Because these regions do not recombine they change very slowly, so they may be useful in identifying stable paternal lineages over thousands of years. Mutations occasionally occur in this DNA, however, which are then inherited down the paternal line.

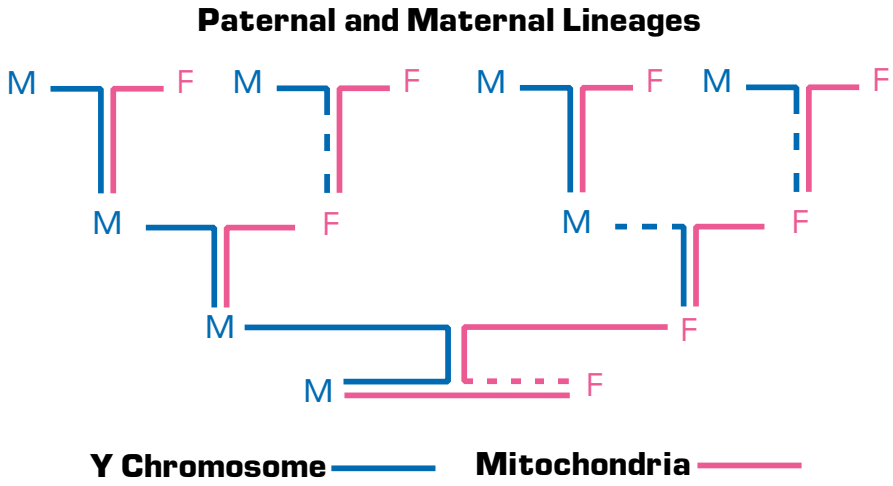


Figure 2. Maternal lineages can be traced through mitochondrial genes, which are inherited by males and females only from the mother. Paternal lineages can be traced through the Y chromosome, which is inherited only by males and only from the father. (M=male and F=female)

Evolution of the Y Chromosome

The evolutionary ancestor of the sex chromosomes was a pair of matched, autosomal chromosomes that acquired sex-determining genes on one member of the pair. This occurred about 350 million years ago in a reptile-like ancestor. Over time additional genes with male-specific functions accumulated in this same chromosome, called proto-Y, which then lost its ability to recombine with its counterpart chromosome, called proto-X. There are four regions of the proto-X chromosome, which appear to have been involved in four different steps, resulting in the loss of recombination with proto-Y. Each of the four regions accumulated mutations in those non-recombining regions of proto-Y at four different times in evolution. Each time recombination was lost there was degradation and loss of the non-recombining region. Over time this chromosome evolved into Y, losing most of its genetic information as a result of the degradation of the non-recombining regions of the chromosome. Its partner chromosome evolved into the X chromosome. The degeneration of the Y was offset at various times by additions of autosomal genes to this chromosome (as well as to X), leading to a pattern of loss and gain of genetic material over a period of about 170 million years (**Fig. 3**).

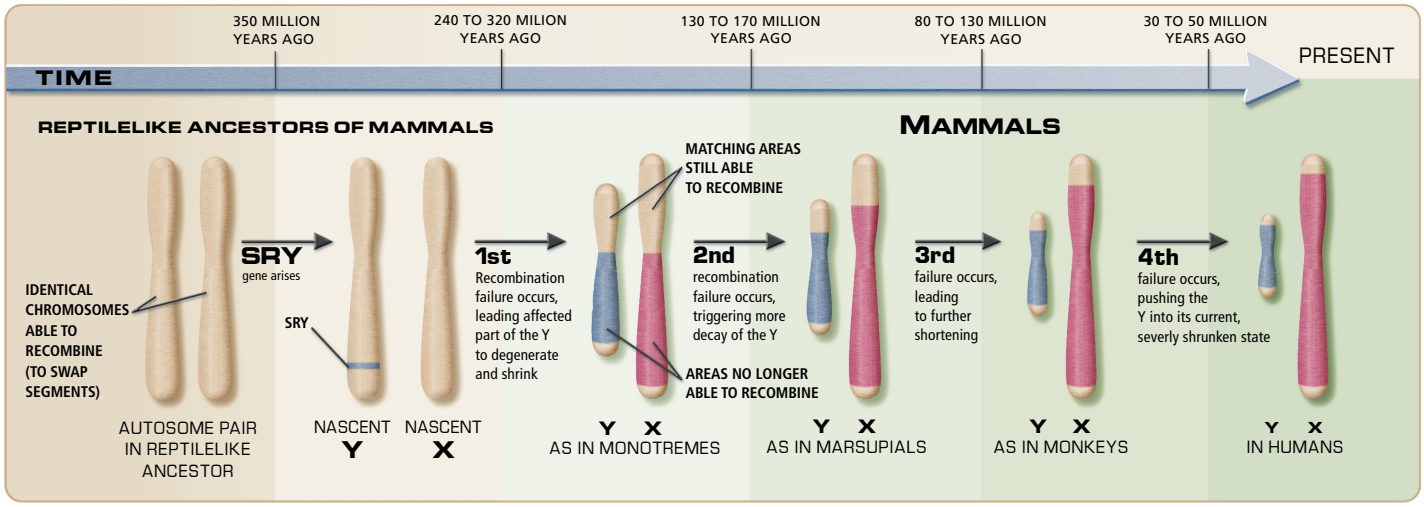


Photo-illustration by Bergmann Graphics

Adapted from: *Scientific American,* February 2001, "Why the Y is So Weird"

Figure 3. The degeneration of the Y occurred in four discrete episodes, beginning about 300 million years ago when a reptile-like ancestor acquired the SRY gene on one of its autosomal chromosomes. Each of the four episodes involved a failure of recombination to occur between the X and the Y chromosomes, resulting in subsequent decay of some genes in the non-recombining region.

X Inactivation

Having a single copy of any chromosome other than the X or the Y is lethal in humans; however, only one X chromosome is needed for normal development to occur. Therefore, the evolutionary process that resulted in a loss of genes from the Y chromosome would seem to have presented a problem. At least two possible mechanisms could balance gene expression between the two X chromosomes in females, versus only one X in males. Gene activity on the one X present in males (relative to the ancestor before the evolution of the XY system) could be increased so that these genes produce twice as much in males as in females. Alternately, X-linked genes could have their activity decreased in females. The first mechanism is seen in some insects, including *Drosophila*, while mammals use a special variant of the second, called **X-inactivation**. In X-inactivation, female embryos randomly inactivate one X chromosome in each cell, resulting in only one functional copy of X-linked genes in both males and females.

X-inactivation requires a locus on the X, called the X-inactivation center. At this locus, inactivation occurs in response to a developmental cue, which is present only at specific stages of embryo development. Inactivation occurs because of a specific type of RNA, which binds to one X chromosome, preventing transcription of the genes on this particular copy. In addition, enzymes add methyl groups to the DNA of the inactive X, resulting in repression of transcription. The inactivated X is visible during interphase in mitosis as a condensed chromosome, called a Barr body. It replicates in the S (synthesis) phase of the cell cycle later than does the active copy. Inactivation of one of the two X copies in a female leaves only one active X chromosome in any cell. An individual who has three X chromosomes has two inactivated copies of the X, producing two Barr bodies.

Because the X is inactivated randomly in cells, one cell could have the maternal X inactivated, while the adjacent cell could have the paternal X inactivated. This causes a pattern of gene expression called **mosaicism**, which occurs when different alleles of X-linked genes are expressed in different cells. A classic example of mosaicism is the female calico cat, which inherits an X-linked allele for yellow coat color from one parent and an X-linked black allele from the other. One or the other color is

expressed in patches of the coat that represent cells descending from parental cells with either an active maternal X or paternal X.

Genetic Imprinting

Some genes are expressed only from the maternal chromosome, while others genes are expressed only from the paternal chromosome. The second gene copy is silenced during gamete formation in the egg (when maternal gene copies are silenced) or the sperm (when paternal gene copies are silenced). This is known as **genetic imprinting**. Imprinting occurs in each generation when new egg and sperm cells are produced.

Relatively few genes in humans are known to be imprinted and such genes tend to be clustered in the genome. The gene imprinting occurs by the addition of methyl groups to the DNA of the silenced gene, preventing transcription of the gene. This gene silencing acts in much the same manner as mutation or deletion of one copy of a gene, except that it is not a permanent, heritable change. If the one remaining active gene is deleted or mutated, there is no extra, functional copy on the second chromosome; therefore, mutation of the single, active copy of an imprinted gene may result in disease. Similarly, as a result of an error, cells may receive all or part of a pair of chromosomes from a single parent. For imprinted genes, that means that the cell receives either two imprinted copies or two active copies. If both copies are imprinted, there is no functional gene. Two active copies of a gene may also result from a mutation that leads to loss of imprinting; neither copy will be silenced. Too many active copies of a gene may result in overexpression of a gene, which can cause disease. A number of cancers have been associated with failure to imprint genes, especially genes that produce growth factors. Overexpression of growth factors can disrupt the cell cycle, contributing to uncontrolled cell growth and cancer. (See the *Cell Biology and Cancer* unit.)

Testis-Determining Factor

The presence of a Y chromosome is usually necessary and sufficient for male development: a 45XO human is female, while a 47XXY is male. It also typically leads to formation of a testis in the mammalian embryo — the primary sex-determining event. The testis then produces and secretes the male hormones, androgens, resulting in the formation of male genitalia. In the absence of Y, the pathway leads to development of a female (**Fig. 4**). Therefore, the Y must contain a testis-determining factor.

The region of the Y chromosome that carries the testis-determining factor contains a gene called SRY (sex region Y). Its product binds to DNA, acting as a transcription factor that is critical for testis production. Scientists studying **sex reversal**, a difference between the chromosomal sex and the phenotypic sex, confirmed the importance of SRY. They determined that infertile males who were XX had all acquired a particular snippet of the Y chromosome, which was translocated to X. That small fragment of the Y carries SRY. Conversely, many XY females have a deletion of the part of the Y that includes SRY. Introduction of the mouse SRY gene into an XX mouse causes the formation of testis and the animal develops as a male anatomically; however, it does not produce sperm. Thus, SRY is the testis-

determining factor, and is the only gene on the Y chromosome that is essential for development of male genitalia. Some genes required for male fertility are on the Y chromosome, while others are on the X or on autosomal chromosomes. The DAZ genes on the Y are essential for sperm formation; deletion of DAZ results in male infertility.

In the first few weeks of development a human embryo develops a sexually indifferent gonad, which can become either a testis or an ovary. Without SRY to stimulate testis development, the gonad becomes an ovary and the embryo develops into a female; the development pathways of both male and female are complex, however, and are regulated by several gene products (**Fig. 4**).

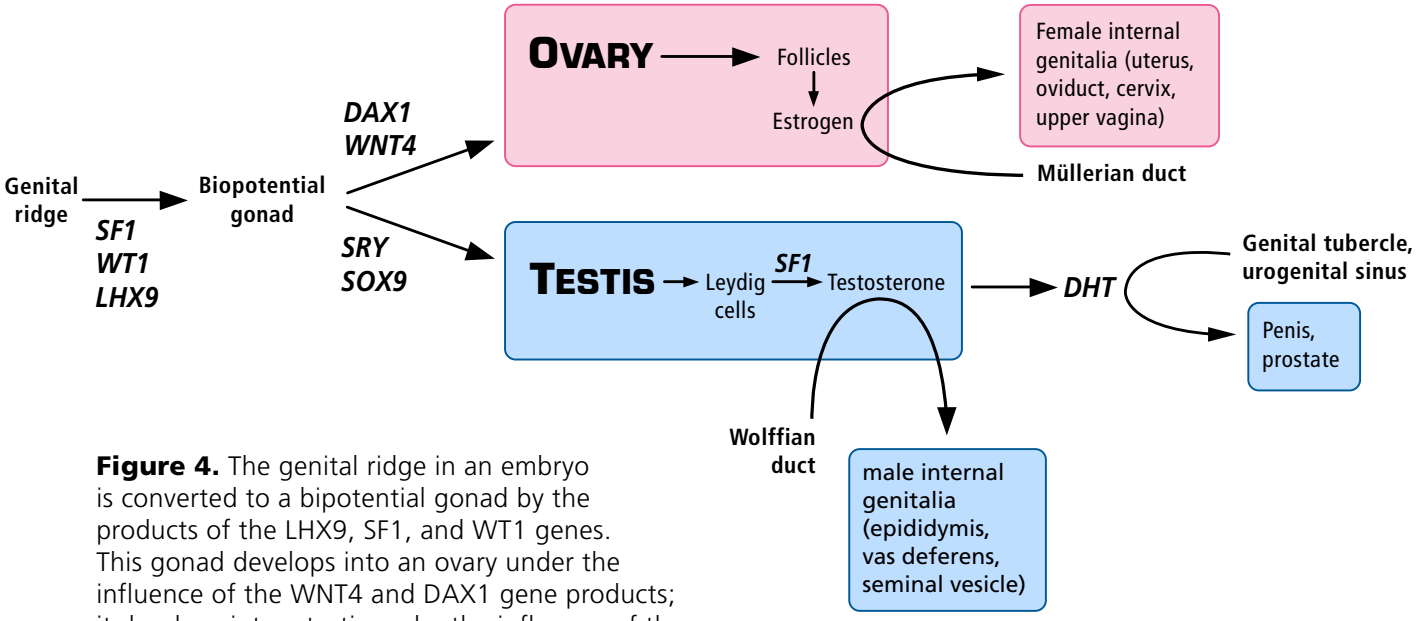


Figure 4. The genital ridge in an embryo is converted to a bipotential gonad by the products of the LHX9, SF1, and WT1 genes. This gonad develops into an ovary under the influence of the WNT4 and DAX1 gene products; it develops into a testis under the influence of the products of the SRY and SOX9 genes. The ovary produces cells that make estrogen, which causes the Müllerian duct to differentiate into the female genitalia. The testis makes two hormones, anti-Müllerian duct factor (AMH), which causes the Müllerian duct to regress; and testosterone, which causes the Wolffian duct to differentiate into male internal organs. Testosterone is also converted into dihydrotestosterone (DHT), which is required for development of male external genitalia.

For example, the product of the DAX1 gene (present on the X chromosome) appears to interact with SRY: an excess of SRY leads to testis formation, while an excess of DAX1 leads to ovary formation. A mutation in DAX1 leads to sterile males but has no effect on females. An extra copy of DAX1 in a male leads to a sex-reversed XY female. An SOX9 mutation (on chromosome 17) in a male leads to sex-reversed XY females, while an extra copy of SOX9 in a female can result in a sex-reversed XX male. Conversely, an extra copy of WNT4, which is implicated in ovary formation, in a male results in a sex-reversed XY female.

Hormones

Hormones are small molecules that bind to specific target cells to modify the response of the cell, usually affecting gene expression. For example, estrogen is a small, hydrophobic molecule that binds to

Table 1. **Genes Involved in Human Sex Determination**

Gene	Function of protein	Action
SRY	Transcription factor	Leads to development of male gonads; XY males lacking SRY show sex reversal to female.
WT1	Transcription factor	Leads to development of male gonads; XY males lacking WT1 show sex reversal to female.
SF1	Transcription factor	Leads to development of male gonads; XY males lacking SF-1 show sex reversal to female.
SOX9	Transcription factor	Leads to development of male gonads; XY males lacking SOX-9 show sex reversal to female.
DAX1	Transcription factor	Leads to development of female gonads; XY males with a duplication of gene for DAX-1 show sex reversal to female.
WNT4	Signaling factor	Leads to development of female gonads; XY males with a duplication of gene for WNT-4 show sex reversal to female.

estrogen receptors. The estrogen-receptor complex then enters the nucleus and binds to specific DNA sequences in certain genes, and turns on or off transcription of those genes.

In females the ovaries secrete estrogens and progesterone, which are essential for the development of female genitalia during fetal development (**Fig. 4**). These hormones are also required for sexual development at puberty, and for pregnancy. The ovaries also produce a small amount of testosterone, although much less than testes in males produce.

In males the testes secrete the major androgen, testosterone. Synthesis of this hormone increases significantly at puberty, when it is responsible for adult sexual development. Androgens are also essential for the development of male genitalia during fetal development (**Fig. 4**). Some testosterone is converted to estrogen in males and is important for bone formation.

Animals in utero can be affected by hormones produced by nearby siblings of the opposite sex. The placement of an animal, such as a mouse in a litter, may have a long-term effect on physiology or behavior. Female mice that develop in the uterus between two males have shorter fertile periods than do females that develop between two females. Male mice prefer to mate with the females that develop in an all-female environment. Females who develop between two brothers in utero are more aggressive towards intruders than are females who develop with two sisters.

Hormones also affect mature adults. Males and females have receptors for estrogens, progesterone, and androgens in various tissues. Transsexuals (individuals who have a conflict between their biological sex and their perceived gender) must take hormones of the opposite

sex if they choose to undergo a sex change. Males can develop breasts, decrease facial hair production, and change the texture of their skin and hair as a result of estrogen and progesterone therapy combined with anti-androgen drugs. Conversely, high levels of testosterone can have a masculizing effect on females. Interestingly, individual differences in natural hormone levels and hormone sensitivity mean that those undergoing a sex change require individualized hormone treatment programs.

Intersex

For some individuals, determination of biological sex can be difficult. Intersex refers to genetically determined differences of the reproductive system. This can include differences in internal reproductive organs, external genitalia, or karyotype. Mild intersex conditions include, in males, a condition in which the urethra opens on the underside of the penis or, in females, an enlarged clitoris. Female intersexuals (karyotype 46XX) (also called female pseudohermaphrodites) have normal ovarian tissue, and have either male or ambiguous genitalia. This is usually a result of a change in the fetal adrenal glands, leading to production of abnormally high levels of androgens. The androgens produce some masculine features in female infants: ovaries and uterus form, but the external genitalia appear male-like. This accounts for about two-thirds of intersex states.

Male intersexuals (karyotype 46XY) (also called male pseudohermaphrodites) have normal testes with female or ambiguous genitalia. They most often result from several different genetic alterations in pathways of testosterone synthesis and metabolism. For example, males who have a mutation in the gene that converts testosterone to dihydroxytestosterone have normal testes but have a very small penis and a vaginal pouch. In gonadal dysgenesis the testes fail to secrete androgens or müllerian-inhibiting hormone, leading to formation of female genitalia. With estrogen treatment, however, these individuals will grow into females. A condition called micropenis results from lack of androgens later in fetal life; testosterone treatment can stimulate masculizing puberty in these individuals.

Androgen insensitivity syndrome (AIS) occurs when a male produces cells that cannot respond to androgen. The defect is in a gene on the X chromosome that produces the androgen receptor. Individuals may have complete or partial androgen insensitivity. In complete AIS the testes develop in the embryo, and produce testosterone and the hormone that inhibits development of female internal reproductive organs (**Fig. 4**). However, because the cells do not respond to testosterone, female genitals develop, which may be incomplete. The newborn appears to be a female and develops external female characteristics at puberty. Lacking internal female reproductive organs, though, the individual with AIS does not menstruate and is infertile. In incomplete AIS, individuals may appear male or female, but there may be abnormalities in the external genitalia. Maria Patino, a Spanish runner with complete AIS, was not allowed to compete in the 1985 World University Games in Kobe, Japan because she failed the gender test. (See the *Sex and Gender* video.) Because of such difficulties in determining sex, the International Olympic Committee abolished gender testing in 1999.

Ethics of Intersex Treatment

Common medical treatments of intersex babies include:

1) assignment of gender based on a variety of clinical tests
2) surgery to remove internal gonads that might become cancerous
3) reconstruction of external genitalia appropriate for the assigned sex
4) if necessary, treatment with appropriate hormones

Unless a penis is present, most intersex babies are assigned female because it is not possible to construct a fully functional penis. The gender assignment and the surgery are usually done immediately after birth, with possible additional surgery after puberty. Today, some physicians such as Eric Vilain (featured in the video) recommend allowing the child to make his or her own surgical decisions later in life. However, most parents choose the surgery earlier because they are uncomfortable with the ambiguity.

The Intersex Society of North America believes that intersex is not an abnormality but rather "an anatomical variation from the standard male and female types."[3] The Society also believes that the decision regarding treatment, if any, should be made by the individual when he or she is capable of informed consent. The Society has two objections to treatment: 1) treatment assumes that intersexuality is a disease, and 2) surgery often damages sexual function, while still failing to produce anatomically normal genitals. They claim that physicians have traditionally failed to communicate to parents the basis for the assignment of gender, which is not always (and in the case of XY individuals without a penis, never) made based on biological sex. They also claim that some physicians have failed to inform parents of alternatives and presented elective surgery as essential for health.

Physicians were once guided by the idea that infants are gender neutral at birth, and that normal gender development would follow from the environment of the child based on the sex assigned to the child.[4] They believed that gender came from "nurture" rather than "nature." In recent years individuals, especially intersexuals, have protested that the environment does not control gender and, given that the treatments are irreversible, they should be delayed until the child determines his or her true gender. Others worry, however, that children with ambiguous genitals will be ridiculed, causing permanent psychological damage that could be avoided.

Homosexuality

There is no simple genetic test to differentiate homosexuals from heterosexuals. However, many studies of siblings have consistently found a much higher incidence of homosexuality in pairs of monozygotic (identical) twins compared to dizygotic (fraternal) twins, strongly suggesting a genetic component to homosexuality. Although there is some suggestive evidence linking variation at specific regions of the genome with the propensity for being homosexual, these studies are not yet conclusive.

Sex and Disease

Sex is an important aspect of human identity, but it is also important in health. Women outlive men. In the United States at the start of the twenty-first century, a woman's life expectancy at birth is 79 years, and

a man's is 72. While other countries have greater or lesser average life expectancies, female life expectancy is still greater in nearly all countries. In fact, for most animals that have been studied, females outlive males; female sperm whales outlive males by thirty years on average. Many factors contribute to this effect, including genes, hormones, and lifestyle factors. Historically, the greatest death risk for women has been childbirth. In developed countries, however, this risk has decreased markedly in the last century, significantly increasing a woman's lifespan.

Males die more often than females — even before birth. Although there are 115 male fertilized eggs for every 100 female, the ratio for live births is 104 males to 100 females. Each year after birth, more males die than females; so, by age 100 there are only 11 males for every 100 females. With improvements in health care, the gap between longevity in men and women is decreasing. However, one troubling factor contributing to the narrowing of the gap is an increase of diseases in women that have been typically considered male diseases, especially cardiovascular disease.

There are two aspects of the longevity gap: Why do men die young and why do women live so long? Hormones appear to be part of the answer to both of these questions. Testosterone may contribute to early death in males. The greatest difference in death rates between males and females occurs during the teen years, when males experience a surge in testosterone. This increase correlates to increases in death in males by accidents, homicide, and suicide; however, these behavior-related deaths continue to contribute throughout life to male mortality more than they do to female mortality.

While teenage females also die from behavioral causes, the incidence is much lower than for males. Female teenagers also experience an increase in hormones; these hormones, however, generally correlate with increased longevity in women. The strongest evidence for the protective effects of female hormones is the increased risk for several diseases after menopause, notably cardiovascular disease and osteoporosis. In males of all ages, testosterone increases the levels of undesirable LDL cholesterol and decreases the levels of the desirable HDL cholesterol, increasing the risk for cardiovascular disease. In contrast, estrogen appears to have a beneficial effect on cholesterol levels. As of 2003, there is much controversy about whether estrogen replacement after menopause gives any significant health benefits for women. In fact, some studies suggest that replacement therapy may do more harm than good.

Women may also enjoy advantages over men in physiology and metabolism, probably because of hormone differences. Women have lower metabolic rates than men, likely leading to less oxidative damage to cells. Oxidative damage results from free radicals, which alter DNA, RNA, and protein in cells. This may explain why oxidative damage is linked to diseases such as cancer, Alzheimer's, and atherosclerosis. In animal studies, lowering metabolism by decreasing calorie consumption has been shown to significantly increase lifespan. In addition, because they menstruate, women have less iron in their blood. (High levels of blood iron are associated with oxidation of LDL cholesterol, which contributes to cardiovascular disease.)

Women also enjoy a genetic advantage because they have two copies of the X chromosome. Mutations in genes on the X chromosome

typically do not cause disease in females because there is a normal copy. Two X-linked diseases are hemophilia and muscular dystrophy. Because X-inactivation occurs randomly in each cell, about half of the cells of women heterozygous for these conditions would be normal. Additionally, the normally inactivate copy of the X chromosome in females (resulting from X-inactivation during development) may be at least partially restored as women age, allowing the inactive X to provide a good copy of a gene that was lost or altered by mutation in the other X chromosome.

One area in which women do not enjoy an advantage over men is in autoimmune diseases. Women are more susceptible to these diseases, such as systemic lupus erythematosus (lupus) and rheumatoid arthritis. There isn't a simple explanation for this increased risk; instead, it appears to result from a combination of genetic, environmental, and hormonal effects.

Lifestyle choices also affect longevity. Early in the twentieth century, men smoked more than women, a factor that is thought to account for much of the gender gap in longevity. As more women began to smoke, the gap decreased. Studies indicate that women smokers may have an increased risk of lung cancer because they have higher levels of an enzyme that produces carcinogens from tobacco smoke. In addition, middle-aged women smokers live no longer than do men smokers, suggesting that smoking eliminates any health advantage conferred by gender.

Despite the evidence for gender-based differences in physiology, metabolism, disease, and response to certain drugs, women were excluded from most medical studies for many years. Why? It wasn't just sexism: the difficulty in controlling the monthly cycles of hormones, and the concerns about possible pregnancy simply made it easier to leave women out of the studies. In 2001 the Institute of Medicine issued the report "Exploring the Biological Contributions to Human Health: Does Sex Matter?" The report concluded that sex was very important in health, and that women should be included in all studies of diseases that could affect them.

With improvements in health care and an understanding of the importance of nutrition and exercise, it is likely that the longevity gap will continue to decrease. We may eventually understand which components of female longevity are the result of sex and which are the result of gender.

References

1) Beale, B. 2001. The sexes: New insights into the X and Y chromosomes. *The Scientist* 15[15]:18.

2) Rozen, S., H. Skaletsky, J. D. Marszalek, P. J. Minx, H. S. Cordum, R. H. Waterson, R. K. Wilson, and D. C. Page. 2003. Abundant gene conversion between arms of palindromes in human and ape Y chromosomes. *Nature*, 423:873–76.

3) http:// www.isna.org/library/dreger-compare.html

4) Money, J., J. G. Hampson, and J. L. Hampson, 1955. Hermaphroditism: Recommendations concerning assignment of sex, change of sex and psychological management. *Bulletin of the Johns Hopkins Hospital* 97:284.

Further Readings

Articles

Beale, B. 2001. The sexes: New insights into the X and Y chromosomes. *The Scientist* 15[15]:18.
Discussion about SRY, DAZ, WNT.

Jegalian, K., and B. T. Lahn. 2001. Why the Y is so weird. *Scientific American,* February, 56–61.
Evolution of the Y chromosome.

Jobling, M. A., and C. Tyler-Smith. 1995. Fathers and sons: the Y chromosome and human evolution. *Trends in Genetics* 11:449–56.

Lewis, R. 2000. Reevaluating sex reassignment. *The Scientist* 14[14]:6.
Evidence supports nature over nurture in establishing gender identity.

Skorecki, K., S. Selig, S. Blaze, R. Bradman, N. Bradman, P. J. Waburton, M. Ismajlowicz, and M. F. Hammer. 1997. Y chromosomes of the Jewish priests. *Nature* 385:32.

The following is a series of articles on sex-based differences in the biology of males and females:

Kreeger, K. Y. 2002. Sex-based differences continue to mount. *The Scientist* 16[4]:26.
When it comes to autoimmunity, the sexes respond in their own ways.

Kreeger, K. Y. 2002. X and Y chromosomes concern more than reproduction. *The Scientist* 16[3]:25.
Clues lie within about which sex is more predisposed to various diseases.

Kreeger, K. Y. 2002. Yes, biologically speaking, sex does matter. *The Scientist* 16[1]:35.
Researchers move beyond the basics to better understand the differences between men and women.

Kreeger, K. Y. 2002. Sex-based longevity. *The Scientist* 16[10]:34.
Societal and lifestyle differences — not biology — appear to have the greatest influences on whether men or women live longer.

Glossary

Androgen Insensitivity Syndrome (AIS). A condition resulting from an alteration in a gene on the X chromosome that normally produces the androgen receptor. This condition leads to a male (XY) with cells that cannot respond to androgens. Because the cells do not respond to testosterone, female genitals develop that may be incomplete.

Genetic imprinting. Differential expression of a gene, depending on whether it was maternally or paternally inherited.

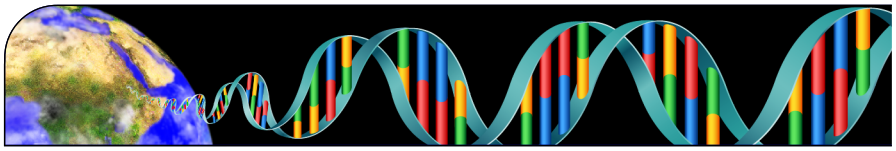
Intersex (intersexual). An organism with external sexual characteristics that have attributes of both sexes. Sometimes used to include individuals whose phenotype cannot be predicted from sex chromosome karyotype.

Mosaic (mosaicism). A tissue containing two or more genetically distinct cell types, or an individual composed of such tissues.

Sex reversal. A discrepancy between an individual's sex chromosomes and their sexual phenotype.

Single nucleotide polymorphism (SNP). Variations in the DNA sequence that occur when a single nucleotide (A, T, C, or G) in the genome sequence is changed.

X-inactivation. Functional inactivation of one copy of the X chromosome in cells of females.



REDISCOVERING BIOLOGY

Molecular to Global Perspectives

Biodiversity

"It is a somewhat sobering thought that we know more about the number and position of stars in our galaxy, places that none of us will ever visit, than we do about the myriad of small animals that live in our backyard. This is despite the fact that these creatures eat our plants, sometimes bite us but most importantly contribute to the cycling of nutrients that sustain life." MARK DANGERFIELD[1]

Alarmed by the rapid deforestation of the species-rich tropical rain forest, prominent environmental biologists such as the Harvard ecologist Edward Wilson became increasingly active during the 1980s, warning the public about the impending crisis of species loss. In 1986 Wilson and others convened the National Forum on Biodiversity to discuss various problems associated with ecosystem loss. Calling attention to the scope of the crisis, that forum's organizers coined a new word: **biodiversity**.

What is Biodiversity and Why Should We Conserve It?

The term "biodiversity" was derived from "biological" and "diversity," and refers to the total diversity of all life in a given locale — one as small as a backyard (or smaller) or as large as the entire planet Earth. One example of a biodiversity measurement is bird watchers listing the species they see in an area on a given day. Although it is often thought of as the number of species in a locale, biodiversity actually has a much wider definition and encompasses levels above and below that of the species. Wilson described biodiversity as the "totality of hereditary variation in life forms, across all levels of organization, from genes to chromosomes within individual species to the array of species themselves and finally at the highest level, the living communities of ecosystems such as forests and lakes."[2]

There is a strong and growing consensus among environmental biologists that we are currently in the midst of a biodiversity crisis. Human-induced global climate change is now accepted as fact. Habitats are rapidly disappearing. Species are going extinct at accelerating rates.

Why should we care about preserving biodiversity? Environmental biologists have outlined two general reasons. First the utilitarian reasons: We rely on a large number of animal, plant, and fungal species for various purposes including food and medicine. In fact, as Simon Levin notes, about forty percent of "all prescription drugs in the

United States contain active ingredients originally derived from nature"[3]. Moreover, our current knowledge is probably akin to the tip of an iceberg compared to the potential medicinal or other benefits from species that remain undiscovered. This is particularly true with respect to microbes and fungi, which we know less about than plants and animals.

In addition to the benefits from individual species, humans also benefit from maintaining healthy ecosystems; perturbing these ecosystems can adversely affect human health. For instance, Lyme disease emerged in the northeast United States because of changes in the forest ecosystem of that region. As the forests became more fragmented, population sizes of white-footed mice soared as they were now free from competitors or predators, whose populations had declined in the now patchy forests. The mice are a source of blood for ticks, which can carry the Lyme-disease bacterium. As the diversity of other small, ground-dwelling rodents decreased, the mice became an increasingly exclusive source of food for ticks, which also feed on humans and other mammals. This resulted in a surge in the exposure of humans to the bacteria. Through a series of links, forest fragmentation has permitted Lyme disease to rapidly become a major health problem in the eastern United States. (See the *Microbial Diversity* unit.)

In addition to the utilitarian reasons, there are also non-utilitarian reasons to preserve biodiversity. Part of the beauty of nature comes from the copious diversity of life. Most would agree that a marked reduction in the Earth's biodiversity would make it a much poorer planet. Related to both the utilitarian and the non-utilitarian reasons is that biodiversity is essentially irreplaceable. The creation of new species by the natural process of speciation usually occurs in time spans of many thousands of generations, far exceeding human lifetimes. The biodiversity that disappears on our watch will be lost not only for our children and their children, but will remain lost for countless generations to follow. In human terms, extinction is forever. Is it moral for humans to cause the irrevocable loss of other species if we can avoid it?

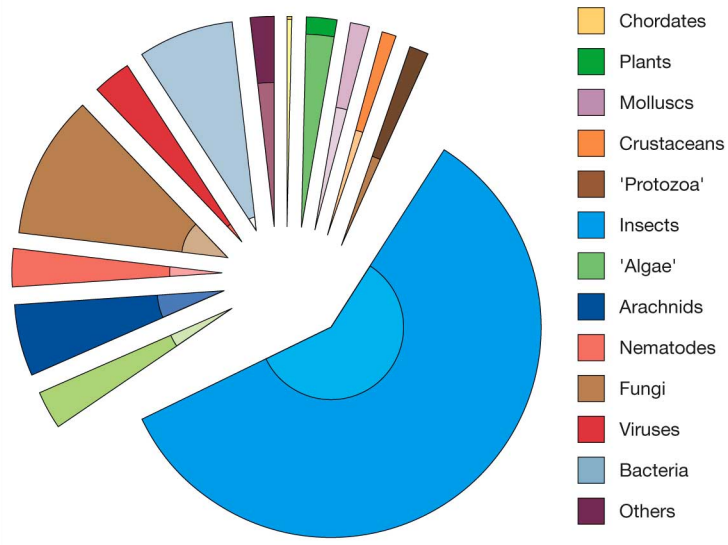
The line between utilitarian and non-utilitarian reasons for preserving biodiversity is blurred. Some reasons now listed as "non-utilitarian" may actually turn out to be utilitarian. Recent research is starting to give us hints that as diversity collapses, the whole ecosystems on which we depend may collapse on a global scale as well. The loss of diversity from a particular area may have a more drastic consequence than simply "it's not pretty anymore" — it may come to mean, "this is now a wasteland of biological life."

Global Species Diversity

Biodiversity is copious and imperiled, yet, it is difficult to measure. This feature makes it also difficult to quantify its loss as well: we know little about what we are losing. Despite its importance, knowledge about biodiversity lags behind that of other areas of science. The statement that opened this chapter echoes those made by several researchers in environmental biology who have been frustrated by the lack of progress quantifying biodiversity. As we shall see, even the simple question "How many species of animals are on Earth?" has not been answered, even to within an order of magnitude.

Before discussing how scientists address the question "How many species of animals are on the planet Earth?" let's first ask, "How many species of animals have been described?" There is uncertainty even to the second question's answer. Some uncertainty reflects differences in opinion among taxonomists about whether different populations are indeed separate species. Some is due to inadequate centralized databases. While there are efforts underway to provide a centralized catalog of described species, none exist as of 2003. The most current estimates are that there are about 1.4 to 1.6 million described species of animals.

What are these 1.6 or so million species of animals? At least one million are insects. A quip from JBS Haldane, polymath and one of the founders of the evolutionary synthesis, illustrates the taxonomic concentration of biodiversity. When asked about what he could divine from nature about the Creator, Haldane replied that he must have had "an inordinate fondness for beetles." Haldane's quip was in reference to the sheer quantity of beetle diversity. There are roughly 450,000 different described species of beetles, representing about 30–40% of known insect species (**Fig. 1**). There are about 200,000 described species of flies. In contrast, there are only about 9,000 species of birds and 4,000 species of mammals. Every year, about 2,400 new species of beetles and 1,200 species of flies are described. Thus, the number of species of beetles scientists will describe in the next five years alone is greater than the total number of current bird species.



From Purvis & Hector, Nature vol. 405 (2000) p 212. Courtesy of Nature Publishing

Figure 1. A pie chart of the hypothesized distribution of species living on earth today.

In addition to animals and plants, biodiversity also includes a vast number of unlabeled species of bacteria, fungi, and protists. These contribute to environmental homeostasis by degrading organic matter and by making the energy in inorganic matter available for growth. Although we often forget these organisms in our consideration of biodiversity, they are critical to the balance and resilience of the environment, especially with respect to their role in nutrient cycles. (See the *Microbial Diversity* unit.)

Much of the known biodiversity is located in the tropics. In general, species diversity greatly increases as one moves toward the equator: specific hotspots of biodiversity are located in tropical rain forests. Even though they account for only about seven percent of the land area on the planet, tropical rain forests are home to around half the known species of animals.

The Erwin Study

Prior to 1982 most biologists thought that the number of undescribed species was roughly comparable to, or perhaps a few times as many as, the number already described. Thus, pre-1982 guesses of the total number of animal species were on the order of several million. But no one really knew.

In 1982 Terry Erwin published a provocative report in which he estimated the number of species of insects to be not several million but an order of magnitude higher — several tens of millions. Erwin reasoned that because the tropical forests appeared to contain vast unexplored areas of biodiversity he would sample there. Erwin, an expert on beetles, fogged the canopy of several trees of the species *Luebea seemannii* with a pesticide. The fogged insects then fell to the ground, allowing Erwin to sample them. As he sampled the beetles, Erwin kept finding new undescribed species. From the canopy of a single species of tree (*L. seemannii*) Erwin found more than 1,100 species of beetles.

How did Erwin arrive at a global estimate for the number of species from his "kill 'em and count 'em" experiment? He first estimated that 160 of those species were specialized to the canopy of that particular species of tree. Considering that beetles represent two-fifths of species diversity of insects, there should be about 400 (160 x 5/2) species of insects specialized to the canopy of *L. seemannii*. This inference assumes that beetle diversity is representative of insect diversity for that species. Erwin assumed that about two-thirds of the insect species were in the canopy and the rest were elsewhere. Based on that assumption, there should be 600 (400 x 3/2) species of insects specialized to *L. seemannii*. There are an estimated 50,000 species of trees in tropical forests. If each tree has 600 species of insects specialized to it, there should be 30 million species of insects in tropical rain forests.

Many authors expressed criticism and reservations about Erwin's extrapolations and inferences. Moreover, there have been only been only a few similar studies, none on the same scale as Erwin's. Much of the criticism revolves around Erwin's initial guess that 160 of the species he collected were specialists. If Erwin had overestimated the proportion of specialists, he would be overestimating the total number of species. Likewise, had he underestimated the proportion of specialists, he would have underestimated the total. Nigel Stork noted that Erwin could well be vastly underestimating biodiversity given that he did not know how much of the diversity of beetles from the *L. seemannii* he had sampled. Suppose Erwin had only sampled one third of the beetle diversity, all of his estimates would be three times too low. Could there be 80 million species of animals? 100 million? In actuality, two decades after Erwin's report, most biologists have revised their estimates for the total number of species downward toward the 10 million range, in part due to studies suggesting that Erwin

overestimated the proportion of specialists. Still, nobody really knows how many species are on Earth.

Another factor that adds to the uncertainty about overall global diversity is our lack of knowledge about smaller organisms. There may be hundreds of thousands or millions of mites and fungi that we have literally overlooked. Even less is known about microbes. There are about 5,000 known species of prokaryotes, but scientists estimate that true diversity could range between 400,000 and 4 million species.

Seven Kinds of Rarity

Biodiversity is not just the number of species in an area. An area that contained twenty species that were all relatively common would be more diverse than one that contained nineteen rare species and one common species. What do we mean when we say a species is rare? Should it just be based on population size? Deborah Rabinowitz proposed that we should consider rarity along three different axes. The first axis is whether the species has a high or a low population size. The second is whether the species has a large or small geographic range. The third axis is whether the species can occur in a broad range of habitats or whether it is restricted to a more narrow range. According to Rabinowitz, a species could be considered common if, and only if, it had a high population size, large geographic range, and occurred in broad range of habitats. All other species were rare. But they could be rare in different senses. Given that there are three binary criteria, there would be two to the third power, or eight, categories with only one being common; thus, there would be seven different kinds of rarity. Rabinowitz used these criteria to classify wild flower species in Great Britain. While thirty-six percent of the species fell into the "common" category, the most prevalent category comprised species that were widely distributed and had high population sizes, but were restricted in their use of habitat. One lesson from this study is that many species that are abundant and widespread may be subject to extinction if their habitat were degraded.

What Factors Determine Extinction Probability?

Other factors being equal, species that have high population sizes are more likely to persist than those with low population sizes. Very small populations are likely to go extinct just by chance in a process called **demographic stochasticity**. As an extreme case, consider a sexual species that has just two individuals. If both members of the pair are the same gender, it is doomed. Even if the pair does include a male and a female, the species cannot persist unless it produces offspring that are of both genders. The risk of demographic stochasticity leading to extinction is most severe for species with population sizes below about 10 but still is a hazard up until a population size of around 50 to 100, especially for species with low birth rates. Compared with sexual species, demographic stochasticity would be less of a factor for asexuals like dandelions because a single individual can reproduce without the need for others.

Species with population sizes that number in the hundreds to a few thousand, while not at risk for extinction due to demographic stochasticity, still face other risks. The random evolutionary force of

genetic drift reduces genetic variation every generation. The strength of genetic drift is inversely proportional to population size. Thus, species with lower population sizes generally have less genetic variation than their more numerous counterparts. Species that have little genetic variation are at risk of being wiped out by disease. They are also less able to respond to other changes such as global warming. Although there is some disagreement, the consensus is that species with populations above 5,000 are probably safe from extinction because of these genetic factors.

Even species with very large population sizes can go extinct. For instance, a species faces extinction if its habitat is lost and it cannot find a suitable replacement. One striking example is that of the passenger pigeon. During the early 1800s the passenger pigeon (**Fig. 2**) had a population size in the billions, on the order of the current human population. Overexploitation by hunters and habitat degradation caused its numbers to rapidly dwindle. As its numbers decreased, the species became vulnerable to the genetic factors listed above and then demographic stochasticity. In September 1914, as World War I was beginning, the last passenger pigeon died in captivity. This species went from very abundant to extinct in a century.

Figure 2. Once a common bird of eastern North America, the last passenger pigeon died in a zoo in 1914.

John J. Audubon, (1829).
Courtesy of Haley & Steele Art Gallery.

Keystone Species and the Diversity-Stability Hypothesis

Not all species are equal with respect to their effects on other species. Starfish feeding in the intertidal zone clean an area free of barnacles and mussels. These barnacles and mussels, without predation by the starfish, would come to dominate the community. In a classic 1966 study Robert Paine removed starfish from enclosures. In those enclosures where the starfish were removed the number of species in the community dropped from fifteen to eight. Paine called starfish a **keystone species**, one whose presence has a dramatic effect on species diversity.

Prior to 1973 most ecologists thought that more diverse ecosystems would be more stable than would ones with fewer species. This general belief, what has become known as the **diversity-stability hypothesis**, was based on a variety of observations but not really tested. One such observation was that cultivated land that had simplified ecological communities was more subject to species invasions than similar areas that hadn't had human influence. In addition, insect outbreaks are much more common in the less diverse boreal forests than they are in tropical forests.

In 1973 Robert May published a theoretical study that challenged the intuitive ideas that ecologists had about the diversity-stability hypothesis. May analyzed randomly constructed communities and found that communities with more species tended to be less, not more, stable. May's study, like more theoretical studies of the 1970s, assumed that population numbers of each species were at equilibria. This assumption was made not because it reflected reality, but because it made the mathematics more tractable. More recent studies have shown that if there is some degree of flux in the population numbers, the community can maintain more species than in equilibrium. This variability may allow different species to respond differently to the environment, and can result in fewer species being lost due to

competitive exclusion. When theoretical ecologists relax the equilibrium assumption and allow for population fluxes, they have found results consistent with the diversity-stability hypothesis: communities with more species are more stable.

Several lines of evidence now support the diversity-stability hypothesis. The studies conducted by David Tilman and his colleagues provide some of the strongest evidence for the hypothesis. In 1982 Tilman divided grassland fields in Minnesota's Cedar Creek National History Area into more than 200 plots. He and his colleagues monitored the species richness and community biomass (the total mass of all plants) in each of those fields over the next two decades. They found that diversity within a community is positively correlated with plant community stability, as defined by the extent of variation in community biomass. Various other studies at different scales have found similar results: stability increases with diversity.[4]

Mass Extinctions

Imagine a meteor ten kilometers wide hitting Earth. The resulting impact would cause ferocious tidal waves and massive earthquakes. Sulfuric acid would be released into the air, leading to intensely acidic rain. Later the atmosphere would dramatically cool because of the dust. The impact would affect nearly all life to some extent, and almost certainly there would be a significant decline in biodiversity.

Re-creation of Chicxulub Impact (2001). Courtesy of NASA.

Figure 3. A re-creation by NASA scientists of the impact made by an asteroid at Chicxulub, on the Yucatan Peninsula. This impact is thought to be the cause of the K/T mass extinction 65 million years ago.

Such a scenario is not just the plot of a Hollywood movie like *Deep Impact*. A meteor that size actually did hit Mexico's Yucatan peninsula sixty-five million years ago (**Fig. 3**). The consequences of the impact led to the extinction of many major groups of animals, most notably the dinosaurs. This mass extinction marked the end of the Cretaceous (K) period and the beginning of the Tertiary (T), and is known as the K/T extinction.

Although the K/T mass extinction is the best known, it was not the largest. That honor belongs to the mass extinction at the end of the Permian period, 250 million years ago. It is often exceedingly difficult to distinguish species in the fossil record, so paleontologists studying extinction usually examine the disappearance of larger taxa (like genera or families). At the end-Permian extinction, sixty percent of families went extinct. Based on the family extinction data, David Raup extrapolated that up to ninety-six percent of species went extinct at this time. Most paleontologists recognize three other mass extinctions, for a total of five (**Fig. 4**).

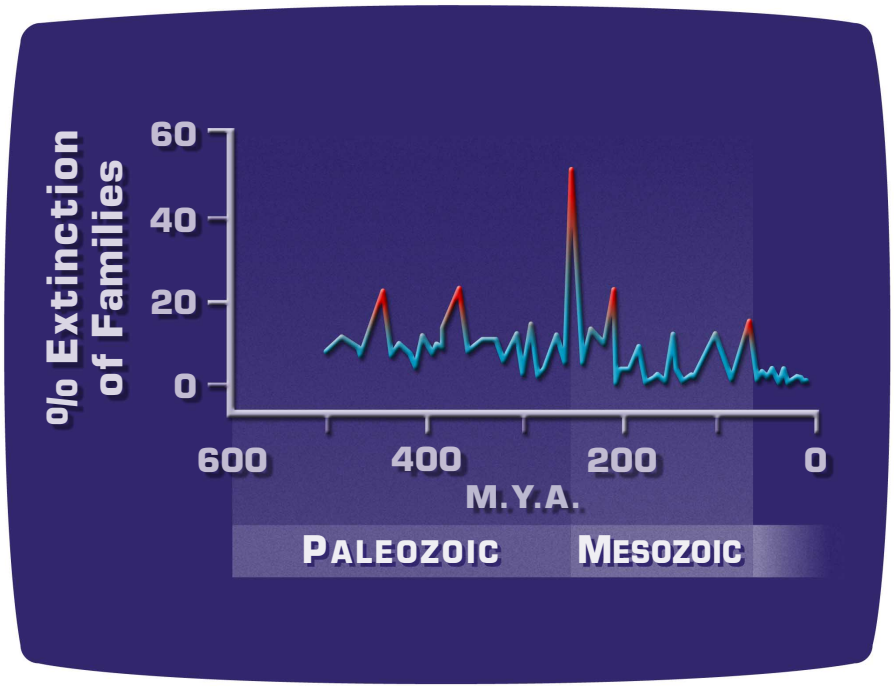
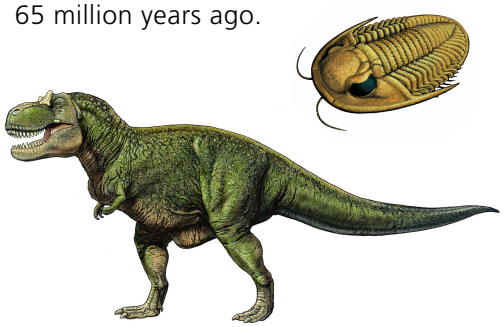


Figure 4. The graph shows an approximate time line of loss of families of species from the earth during the five so-called "mass extinctions." Below, the trilobite was a victim of the extinction at the end of the Permian period, and the familiar Tyrannosaurus rex died out with the K/T extinction 65 million years ago.



Carl Buell (2003). Courtesy of the artist.

Although these mass extinctions happened during a short period by geological scales, they were not instantaneous. In fact, the extinctions probably actually occurred over a period of a few million years.

What were the causes of the mass extinctions? We know the most about the asteroid-caused K/T extinction. Based on changes in the floral composition around the K/T boundary, some paleobotanists have speculated that there was global cooling after the extraterrestrial impact. Oceanic cooling may have led to the disappearance of reef-building organisms. We know less about the other extinctions, but it likely that they were marked by periods of global climate change as well.

Species extinctions during mass extinction events account for only a few percent of total extinctions. Indeed, some paleontologists have wondered whether there is anything special about mass extinction events. Species extinctions occur often but at different rates across time. Perhaps mass extinctions are merely the tail-end of the distribution of extinction rates.

The Sixth Mass Extinction

Should we consider, as some environmental scientists have, that the current biodiversity crisis is the start of a sixth mass extinction? Regardless of how one answers that question, it is clear that we are losing species at rates that, while exceedingly difficult to calculate, are above the background extinction rate and far exceed the speciation rate. Estimates are that 100,000–500,000 species of insects will go extinct in the next 300 years. The higher end of that estimate is comparable to the magnitude of the loss of species during the previous mass extinction episodes. Even the lower estimate represents a considerable loss of biodiversity. Moreover, 300 years is much shorter than the duration of those mass extinction periods.

The current biodiversity crisis stems from several causes; the two major contributors are habitat destruction and global climate change, both of which are largely due to human activity. As discussed earlier, much of the (largely unexplored) biodiversity lies in the tropics and, in particular, tropical rain forests. Tropical forests are being lost at an alarming rate. Conservative estimates place the loss of rain forest during the 1980s and 1990s at about 0.8% per year. This is in large part due to changes in the way the land has been used. For quite a long time, many areas had practiced slash and burn agriculture. In recent decades, however, the practice of cutting and clearing has been used increasingly for grazing or timber harvest, resulting in the loss of the tropical forest habitat. As a consequence, countless thousands of species (most of which are unknown to humans) are imperiled.

Global climate change has also impacted biodiversity. During the twentieth century, the mean temperature has increased by slightly more than one degree Fahrenheit (0.6 degree Celsius), and most of that change occurred between 1970 and 2000. Projections vary between x and y degrees Fahrenheit increase by mid-century. These changes do not appear great in the context of daily and seasonal temperature fluctuations, but they are large in comparison with prehistoric climate changes. While the magnitude of these changes is not beyond the range of historical variation, the rate at which the change has taken place appears to be so. The climate change is human induced, due mainly to increases in carbon dioxide and other "greenhouse gases" that have appeared since the Industrial Revolution and accelerated during the twentieth century.

The human-induced global climate change is coupled with other climate cycles of various temporal and spatial scales. For example, the eastern United States had a cold winter in 2002–3 after several mild winters. In contrast, the western United States had a milder than normal winter that year. The pattern in 2002–3, most likely due to El Niño, does not invalidate the global upward climb in temperatures over a decades-long timespan. In addition to a mean increase in temperature, human-induced global warming is also likely to cause increased variation in climate. Some climate models suggest that the global warming may actually cause the northeastern United States to be cooler. The reason for this seemingly paradoxical possibility is that warming of the oceans could cause the Gulf Stream to be diverted south and east. Were this to happen, it would cause the Atlantic coast to be cooler. Regardless of the specifics of the local changes, more extreme weather will likely exacerbate already fragile ecosystems.

A paper published by Terry Root and her colleagues in 2003 shows that many species have altered their geographic ranges, presumably as a result of global climate change.[5] Of those species that had altered their range, eighty percent were in the direction predicted by climate change models. The mean change of movement was about six kilometers per decade. In addition, many bird species have started laying eggs earlier in the spring. This study shows that forces of small, sustained change can be powerful over long enough time scale. But what about species that are unable to move? What will happen as their habitat changes due to human-induced global climate change?

Because of human-induced climate change and habitat destruction, we face a grave and growing crisis. Biodiversity is being lost at alarming but unknown rates. Moreover, if the diversity-stability hypothesis is true, loss of some species may trigger the loss of others, leading to a vicious circle. Although our knowledge about biodiversity and the extent to which it is lost is too meager, the consequences are too grave to continue in ignorance.

References

1) http://www.bio.mq.edu.au/KCBB/AnnualReport/1998.pdf

2) Wilson, E. O. 1994. *Naturalist*. Washington, D. C.: Naturalist Warner Books, 359.

3) Levin, S. A. 1999. *Fragile dominion: Complexity and the commons*. Cambridge, MA: Perseus Books, 6.

4) McCann, K. S. 2000. The diversity-stability debate. *Nature* 405:228–33.

5) Root, T. L., J. T. Price, K. R. Hall, S. H. Schneider, C. Rosenzweig, and J. A. Pounds. 2003. Fingerprints of global warming on wild animals and plants. *Nature* 421:57–60.

Further Reading

Books

Dobson, A. P. 1998. *Conservation and biodiversity*. New York: Scientific American Library.
An accessible summary about biodiversity from one of the leaders of the field.

Levin, S. A. 1999. *Fragile dominion: Complexity and the commons*. Cambridge, MA: Perseus Books.
Discussion about ecology, complexity theory and approaches to modeling complex systems.

Reaka-Kudla, M. L., D. E. Wilson, and E. O. Wilson (eds). 1997. *Biodiversity II: Understanding and protecting our biological resources*. Washington, D. C.: Joseph Henry Press.
A multiauthored volume on various aspects of quantifying and preserving biodiversity.

Wilson, E. O. 1994. *Naturalist*. Washington, D. C.: Naturalist Warner Books.
The autobiography of a prominent ecologist and one of the founders of the field of biodiversity.

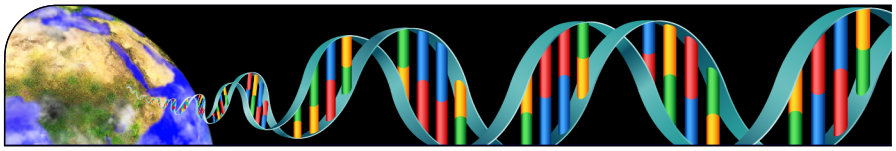
Glossary

Biodiversity. The total diversity of all life in a given locale.

Demographic stochasticity. Variation in numbers or genders of offspring via chance. When population sizes are low, these chance factors can lead to extinction.

Diversity-stability hypothesis. Communities that contain more species will vary less through time in response to various disturbances.

Keystone species. A species whose presence has a dramatic effect on the persistence of other species.



REDISCOVERING BIOLOGY

Molecular to Global Perspectives

Genetically Modified Organisms

"And God said…let them have dominion over the fish of the sea, and over the fowl of the air, and over the cattle, and over all the earth, and over every creeping thing that creepeth upon the earth." GENESIS 1:26 THE HOLY BIBLE

"The Earth does not belong to us. We belong to the Earth."
CHIEF SEATTLE

Introduction

Humans purposefully manipulate the evolution of other organisms. For thousands of years farmers have used selective breeding to improve their livestock and crops. As a result, we have cows that produce more milk, hens that lay more eggs, sheep with better wool, and disease-resistant plants with higher productivity. Another striking example of humans altering other organisms is the great diversity of dog breeds, from the toy poodle to the Great Dane.

Although humans have been manipulating organisms for millennia, genetic engineering simplifies and targets manipulations in an unprecedented way. Transgenic plants and animals are generated with characteristics that cannot be obtained using traditional breeding. Unlike organisms generated by selective breeding, **transgenic organisms** (also known as "recombinant organisms") by definition contain genes from other species. Genetic engineering techniques are used to generate **recombinant DNA**, which contain sequences from different organisms. This DNA then becomes incorporated into a host so that it can be passed to subsequent generations. For example, **Bt** corn expresses a gene for an insecticidal toxin that was "donated" by the bacterium *Bacillus thuringiensis*.

The use of recombinant organisms has become commonplace. For example, bacteria produce human insulin and hepatitis vaccines, and some crop plants are cultivated to be resistant to certain herbicides and insects. There are also transgenic livestock that produce human proteins, such as antithrombin III. The economic value of such products drives research. How did it start? Where is it going? What are the challenges and the risks? In this unit we will explore these questions as they relate to modifying bacteria, plants, and animals.

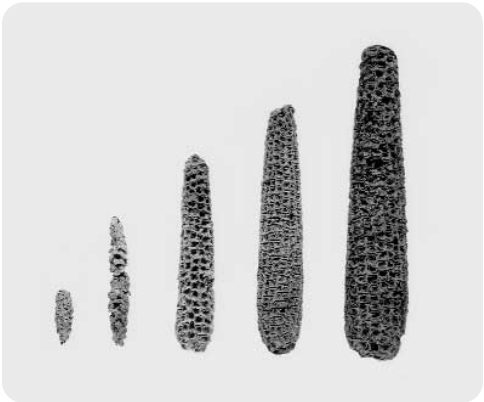


Figure 1. The ancestor of modern corn had tiny kernels, each protected by a tough husk. Domestication of maize, which began thousands of years ago, selected for large sheathed cobs containing large kernels without husks.

Genetic Modification of Bacteria

Bacteria — the first organisms to be genetically engineered — are used for replicating and altering genes that are subsequently introduced into plants or animals. Bacterial systems lend themselves to genetic manipulation in part because of their rapid reproduction rates. It is easy to produce a genetically identical population — a **clone** of bacteria — all containing the gene of interest in a short period. The cells can then be lysed and DNA can be isolated in short order. Bacteria are routinely used to produce non-bacterial proteins. An example is the production of purified proteins for vaccine use. Such proteins can be safer and as effective as vaccines that contain killed or attenuated (weakened) pathogens. Genetic engineering can also produce extensive changes in the bacterium's metabolism. For example, bacteria can be provided with several genes encoding enzymes that allow the production of fuel alcohol from wood.

Researchers have taken advantage of nature to modify bacteria. **Plasmids** are small, circular, self-replicating, extrachromosomal pieces of DNA that occur naturally. A plasmid can encode a protein that offers its host a selective advantage. For example, a plasmid that encodes an antibiotic allows its host bacterium to thwart competing microbes. Alternately, a bacterium might possess a plasmid that encodes antibiotic resistance. Plasmids are readily isolated from bacterial cells and can be altered in vitro by inserting or deleting specific sequences of DNA. Because they can be used to create clones of genes, plasmids are called **cloning vectors**.

Getting the Plasmid In

In nature bacteria have various enzymes that cut up the DNA of their natural enemies, such as bacteriophages (bacterial viruses). Researchers have taken advantage of these so-called **restriction enzymes** to splice DNA for use in engineering bacteria. Hundreds of restriction enzymes have been isolated and each will cut a DNA strand at a specific sequence of nucleotides. Some restriction enzymes generate blunt ends, cutting across both strands of DNA. Others generate a staggered cut, producing "sticky ends." These ends anneal by hydrogen bonding to similar ends on another DNA segment cut with the same restriction enzyme.

Cloning a gene involves identifying a gene of interest in an organism, isolating DNA from that organism, and then using a restriction enzyme to snip the gene from the DNA strand. The gene-containing segment can then be spliced into a plasmid cut by the same restriction enzyme. The bacteria take up the plasmid and are allowed to replicate.

Ordinarily, bacterial cells do not readily take up plasmids. Researchers can use various tricks, however, to get cells more ready to do so. One common method holds the cells on ice in a solution of calcium chloride. The cells are then briefly heat shocked so the plasmid can cross the plasma membrane. An alternate method, **electroporation**, uses a short electrical pulse to open pores in the plasma membrane, allowing the plasmid to pass through.

Marker genes, such as genes for antibiotic resistance, are often engineered into plasmids. These marker genes enable researchers to know which bacteria have the plasmids. The antibiotic is added to the media used to grow the bacteria. Cells that do not contain the plasmid

will fail to reproduce. In addition to marker genes, plasmids typically contain one or more genes of interest. For example, a protein not otherwise expressed by the recipient cell might be produced only when the plasmid is present. Individual colonies of bacteria, each derived from a single cell, can be evaluated for the expression of such novel gene products.

Protein production can be straightforward if the source of the novel gene was another bacterium. However, the goal of modifying bacteria might be the production of proteins encoded by eukaryotic genes from fungi, plants, or animals. This presents challenges. Eukaryotic DNA contains both **exons** (coding sequences) and **introns** (intervening sequences). In eukaryotic cells this DNA is used as a template for the production of mRNA, which must then undergo **mRNA splicing**. Introns are removed and exons are joined to form the mRNA, which travels to the ribosome for protein production. Bacteria lack the enzymes necessary for mRNA splicing, so introducing a eukaryotic gene into bacteria requires a special procedure. First, DNA must be generated that is complementary to the already spliced mRNA. The enzyme **reverse transcriptase** is then used to generate a double-stranded DNA molecule called cDNA, using the mRNA as a template. Finally, this cDNA is incorporated into the cloning vector.

Expressing eukaryotic genes in bacteria presents other problems. After proteins are assembled in eukaryotic cells they are often modified. (See the *Proteomics* unit.) For example, various sugars may be attached to the polypeptide so that glycoproteins are formed. Bacteria are generally unable to accomplish such post-translational modifications, and eukaryotic genes expressed in bacteria may not function properly. The inability of bacteria to perform such modifications has driven scientists to use yeast (*Saccharomyces cerevisiae*) and eukaryotic cell culture to produce some recombinant products.

Are Recombinant Bacteria Safe?

Concerns about the safety of recombinant bacteria were voiced as the technology was developed. Some fear that new, untreatable human pathogens could be inadvertently generated. In 1974 prominent researchers self-imposed a moratorium on certain experiments until they could assess the hazards. After much discussion, the researchers developed biological containment procedures. These include generating recombinant DNA only in bacteria that have mutations to prevent them from surviving outside of the laboratory. The release of recombinant microbes into the environment remains controversial.

Genetic Modification of Plants

New traits introduced to crop plants by genetic engineering have the potential to increase crop yields, improve agricultural practices, or add nutritional quality to products. For example, transgenic crop plants capable of degrading weed killers allow farmers to spray weeds without affecting yield. Use of herbicide-tolerant crops may also allow farmers to move away from preemergent herbicides and reduce tillage, thereby decreasing soil erosion and water loss. Transgenic plants that express insecticidal toxins resist attacks from insects. Crops engineered to resist insects are an alternative to sprays, which may not reach all parts of the plant. They are also cost effective, reducing the use of synthetic insecticides. Genetic engineering has also been used to

increase the nutritional value of food; "golden rice" is engineered to produce beta-carotene, for example. Edible vaccines, present in the plants we eat, may be on the horizon.

The new traits expressed in such transgenic plants are derived from a variety of other organisms. Scientists have given a gene from the bacterium Salmonella to cultivars of soybeans, corn, canola, and cotton to degrade the pesticide glyphosphate (Roundup™). The gene for the insecticidal toxin in transgenic cotton, potato, and corn plants comes from the bacterium Bacillus thuringiensis (Bt). One of the genes allowing vitamin A production in golden rice is derived from the bacterium Erwinia uredovora; others are from the daffodil.

The development of golden rice involved the introduction of several genes into a plant to provide a multistep biochemical pathway. (**Fig. 2**) Rice grain, which serves as a food staple for much of the world, lacks vitamin A. An estimated 100 million to 200 million children worldwide have vitamin A deficiency, a condition that causes blindness; and increases susceptibility to diarrhea, respiratory infection, and childhood diseases such as measles. Beta-carotene and other carotenes (the red, yellow, and orange pigments found in carrots and other vegetables) are the precursors of vitamin A. Rice synthesizes beta-carotene in its chloroplasts but not in the edible seed tissue.

Ingo Potrykus and his colleagues found that geranyl geranyl diphosphate (GGPP), a precursor to carotenoid production, is present in rice seed. They genetically engineered golden rice to express the enzymes necessary for the conversion of GGPP to beta-carotene. The synthesis of beta-carotene from geranyl geranyl diphosphate requires four biochemical reactions, each catalyzed by a different enzyme. A bacterium, *Agrobacterium tumefaciens*, containing three plasmids, was used to introduce all the genes necessary for the complete biochemical pathway for beta-carotene production. It was possible to use three enzymes instead of four because the bacterial enzyme phytoene desaturase accomplishes what two plant enzymes (phytoene desaturase and beta-carotene desaturase) do.

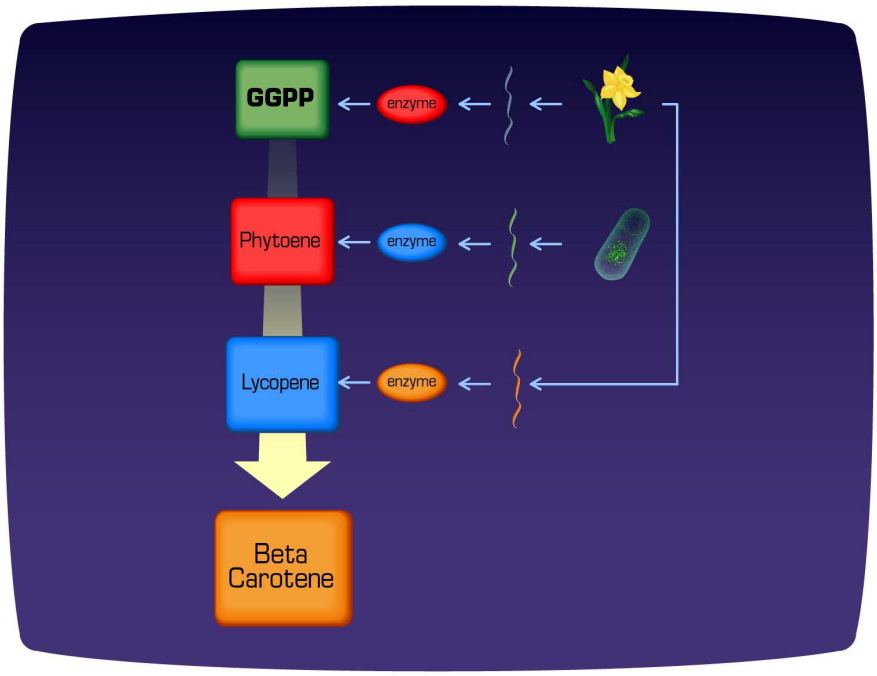


Photo-illustration — Bergmann Graphics

Figure 2. The biochemical pathway for beta-carotene synthesis in "golden rice."

If transgenic plants can help prevent vitamin deficiencies, can they also produce vaccines? Edible vaccines available in crops could help people in developing nations where transportation, refrigeration, and disposable needle supplies are limited. Hugh Mason and his colleagues (Boyce Thompson Institute) have expressed a gene that encodes an *E. coli* protein in potatoes. Volunteers who ate raw, modified potatoes developed antibodies to the protein. Research is underway to see whether the antibodies will protect against diarrhea induced by disease-causing *E. coli.*

Techniques Used for Generating Transgenic Plants

As with bacteria, the ability to genetically modify plants depends on obtaining genetically identical populations and readily manipulating DNA. How do you "clone" a plant? Many plant species naturally undergo asexual reproduction by fragmentation, where segments from a parent plant regenerate a new plant. It is also possible to grow plants in culture from small explants. Another method is to culture plants from **totipotent** cells found in plant meristems. These plant cells can divide and differentiate into the various types of specialized cells. In a test tube, plant cells will divide and form an undifferentiated callus. When hormones in the culture medium are adjusted, the callus will sprout shoots and roots and eventually develop into a plantlet that can be transplanted to soil. To clone a plant — perhaps a plant with new genes — the growing callus is simply subdivided. Thousands of genetically identical plants can be generated in this way.

How do you get a plant to take up a gene? Researchers working with rice often use the soil bacterium *Agrobacterium tumefaciens.* This bacterium, the cause of crown gall disease in many fruit plants, is well known for its ability to infect plants with a tumor-inducing (Ti) plasmid. A section of the Ti plasmid, called T-DNA, integrates into chromosomes of the plant. Recombinant DNA can be added to the T-DNA, the gall-inducing genes removed, and infection by the bacteria — containing the recombinant plasmid — will provide for transfer of novel genes to plant embryos.

Although *Agrobacterium tumefaciens* works for introducing plasmids into rice, not all plants are equally susceptible to this bacterium. Researchers interested in modifying crops such as wheat and corn have turned to other methods for delivering genes to plant cells. One approach is to use a "**gene gun**," (**Fig. 3**) which fires plastic bullets filled with DNA-coated metallic pellets. An explosive blast or burst of gas propels the bullet toward a stop plate. The DNA-coated pellets are directed through an aperture in the stop plate, and then penetrate the walls and membranes of their cellular targets. Some projectiles penetrate the nuclei of cells, where occasionally the introduced DNA integrates into the DNA of the plant genome. Transformed cells can then be cloned in culture.

Marker genes are often included in DNA constructs so that plants that have acquired the novel DNA can be selected. In plants, marker genes include those for herbicide resistance. Plants that grow in the presence of the herbicide are assumed to possess the transgene of interest. The transgenic plant embryos are cultivated in tissue culture. Once mature plants are obtained they are evaluated for the activity of

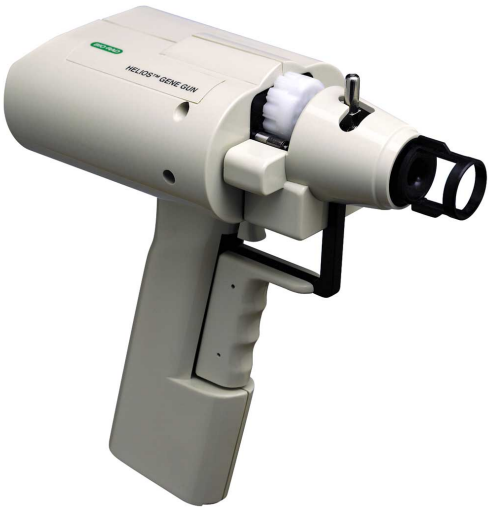


Figure 3.
A "gene gun."

the introduced gene, any unintended effect on plant growth, and product yield and quality. The ability of the gene to be expressed in subsequent plant generations is also evaluated.

Not all genes are expressed in every tissue of a plant. When golden rice was developed it was necessary to ensure that the novel genes were expressed in the endosperm of the seed. The endosperm of a seed is the starchy component that provides energy and nutrients for the developing plant embryo. Regulatory DNA sequences upstream from the specified genes were introduced into the recombinant Ti plasmids. Such regulatory regions influence where and when a gene will be expressed. (See the *Genomics* unit.) The regulatory regions chosen for golden rice provide an uninhibited transcription of the genes in endosperm.

Problems and Concerns

Several concerns have been raised regarding transgenic crop plants. Foremost is the possibility that the process of genetic engineering might inadvertently generate new allergens or toxins that could affect human health. Another concern is that introduced genes from engineered crops could move to other organisms in the environment. Other concerns relate to cultivars that are engineered to produce insecticides. The potential development of insecticide resistance in target pests is worrisome; as is the possibility that non-target, beneficial insects might be affected by engineered plants.

A particular concern is the possibility that transgenic crop plants could affect human health by expressing unanticipated allergens. In March 1996 researchers at the University of Nebraska showed that an allergen from Brazil nuts had been transferred into soybeans. Individuals sensitized to Brazil nuts make antibodies (IgE) specific to certain proteins in the nuts. Engineered soybeans reacted with such antibodies in vitro. Had allergic individuals consumed the transgenic soybeans they would have likely experienced IgE-mediated reactions, ranging from itching to anaphylaxis.

Obviously, expressing a known allergen in food crops is unwise. However, it is difficult to predict whether a protein expressed in a novel organism will cause allergies. A protein isolated from its native species may differ from the same protein (with an identical amino acid sequence) harvested from a transgenic organism expressing that protein. Sometimes sugar or acetyl groups are added to proteins after they are manufactured at the ribosome. The forms of sugar or acetyl groups may vary between organisms. Sugar groups on proteins have been associated with allergenic and immunogenic responses. Hence, allergenicity studies ought to be carried out on the actual material derived from transgenic plants themselves, rather than on just the bacterial proteins. Such studies are not always done.

Critics are worried that engineered plants might generate toxins as a result of the DNA-insertion process. They note that the insertion of genetic material (using gene gun technology, for example) is semi-random, and that the amount and location of DNA inserted into the chromosome varies. If an insert disrupts a regulatory region that serves to “turn off” the production of a toxin, the result might be an over-expression of toxin. Another concern is the inclusion of regulatory regions as part of genetic constructs: the regulation of host genes near an insert could be dramatically affected.

Significant concerns relate to the impact that genetically modified plants could make on the environment. In experiments, transgenic crops are known to hybridize with closely related species. The probability that transgenic traits, as well as other accompanying changes in traits, will show up in wild plant relatives is increasing as genetically modified crops are established. Herbicide-tolerant weeds can evolve; glyphosphate- (Roundup™) resistant rigid ryegrass, for example, has developed only recently. Genetically modified crops must be monitored to reduce unintended degradation of natural ecosystems.

Crops engineered to produce insecticides, such as Bt toxin, bring other concerns. The widespread planting of Bt corn and other crops can result in insects evolving a resistance to Bt toxins. At least ten species of moths, two species of beetles, and four species of flies already have developed resistance to Bt toxins under laboratory exposure. Bt toxins administered as a spray are present only transiently. However, transgenic crops continuously express the insecticidal protein. This ongoing exposure may be more likely to select for resistant insects.

The emergence of a resistant insect population is likely whenever a pesticide is used. One strategy for delaying the emergence of insects resistant to Bt toxin is to plant a "refuge" of conventional crops near Bt-expressing crops. The idea is that these conventional crops will harbor susceptible insects that will mate with resistant insects, diluting out recessive resistance alleles. Of course, if resistance develops as a dominant allele, this strategy will not work.

There are hundreds of known subspecies of *Bacillus thuringiensis*, and the insecticidal toxin derived from each is poisonous only to certain species of insects. Nevertheless, there are concerns that plants expressing genes for such toxins could affect non-target insect species. Some of these species may be beneficial, such as those that provide pollination or consume pests. Laboratory experiments suggest an increased mortality of Monarch butterflies that ingested Bt corn pollen. How frequently this occurs in the field is unknown, and not all laboratory studies have given similar results. The Environmental Protection Agency (EPA) requires toxicity tests on a standard set of organisms before a pesticide can be registered. As of December 2002 the EPA had not demonstrated toxicity of Bt to non-target species. Data gathering continues.

Genetic Modification of Animals

Dolly the lamb stole the headlines as the first example of livestock cloned from DNA of an adult animal. But the real breakthrough came with Polly, the first transgenic lamb. Born the year after Dolly, Polly was given a human gene that encodes blood-clotting factor IX, the protein missing in people with one form of hemophilia. Harvesting such proteins from transgenic livestock is one goal of this research. The road to Polly and subsequent transgenic animals began with research using genetically altered mice. Along the way, technologies for cloning animals, modifying DNA, and targeting expression of proteins to specific tissues were developed. Someday, human gene therapy — supplying genes to patients with missing or altered proteins — may become common practice. However, significant challenges remain. Moreover, risks and ethical concerns must be addressed.

Antithrombin III (AT-III) is an example of a pharmaceutical produced in transgenic livestock. A normal level of AT-III keeps the formation of blood clots under control. Patients with AT-III deficiency may have thromboembolic problems beginning in early adulthood, particularly clots in the legs and pulmonary embolism. Providing therapeutic AT-III can reduce clotting risks in such patients. Other therapeutic proteins being considered for production by transgenic animals include human hemoglobin, human serum albumin, tissue plasminogen activator (used to treat stroke), human alpha-1-antitrypsin (alpha-1-antitrypsin deficiency can cause life-threatening emphysema), various vaccine proteins, and monoclonal antibodies.

For some time, mice have been genetically altered to exhibit human genetic disease. To generate such animal models normal genes in mice are inactivated using "knockout" technology, or altered by replacement of the normal gene with a mutated counterpart. Mouse disease models now exist for cystic fibrosis, beta-thalassaemia, atherosclerosis, retinoblastoma, and Duchenne muscular dystrophy. Such animal models allow researchers to test therapeutic compounds and study the molecular basis of given diseases.

Knockout technology, as well as other genetic engineering approaches, depends on the ability to target genes for insertion into particular locations within the host chromosome. To do this, a region on the chromosome is identified and DNA homologous to that region is engineered into a cloning vector. The newly inserted sequence can then be disrupted by insertion of a selected gene; for example, a marker gene encoding antibiotic resistance. Once cells take up the DNA, homologous recombination on either side of the marker gene allows it to be precisely inserted into the chromosome. At the same time, some or all of the target gene on the chromosome is deleted. (**Fig. 4**)

Gene knockout in pigs is being studied as an avenue for transplanting animal organs into humans. A major cause of tissue rejection is an immune reaction to the carbohydrate galactose-a-1,3,-galactose on the surface of non-human cells. Deletion of the a-1,3,-galatosyltransferase gene may allow the production of animals lacking this surface marker.

As researchers recognized the potential of transgenic livestock for the production of human therapeutics and transplant tissue, farmers recognized the contributions that genetic engineering might make to the economics of livestock production. Cows might be produced that could grow more muscle mass, require less feed, produce more milk, or be leaner. The composition of milk could be changed; for example, casein could be over-expressed to provide increased cheese production. Lactose might be removed from milk for lactose-intolerant consumers. Disease resistant animals could reduce the use of antibiotics. Poultry with less fat content and eggs with lower cholesterol are other goals.

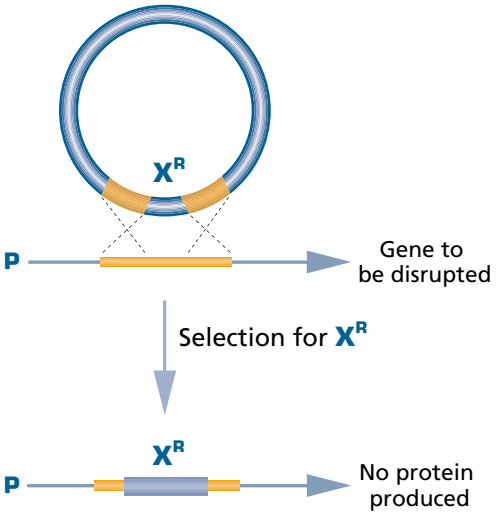


Figure 4. The plasmid contains a gene interrupted by a marker gene (XR). Recombination involving two crossovers between the plasmid and wild type chromosomal DNA with the interrupted gene and the selectable marker.

Cloning Animals

Asexual reproduction in bacteria and plants allows scientists to obtain genetically identical populations; this does not occur naturally in vertebrates, except in twins. In 1996 Dolly the lamb was born: chromosomal material derived from an adult sheep was used to generate an animal with chromosomal DNA identical to that of the donor animal. Cloning livestock, using the techniques that generated

Dolly, may become an economical method for traditional breeders to replicate their superior animals and provide them to farmers. Rather than selling semen, breeding companies might distribute cloned embryos for implantation into surrogate cows. Because Dolly did not possess foreign DNA she was not transgenic. However, she did represent a valuable step toward the development of transgenic livestock. With donor DNA for cloning derived from cultured recombinant cells, it becomes possible to carry out specific genetic modifications and introduce the modified genes into animals.

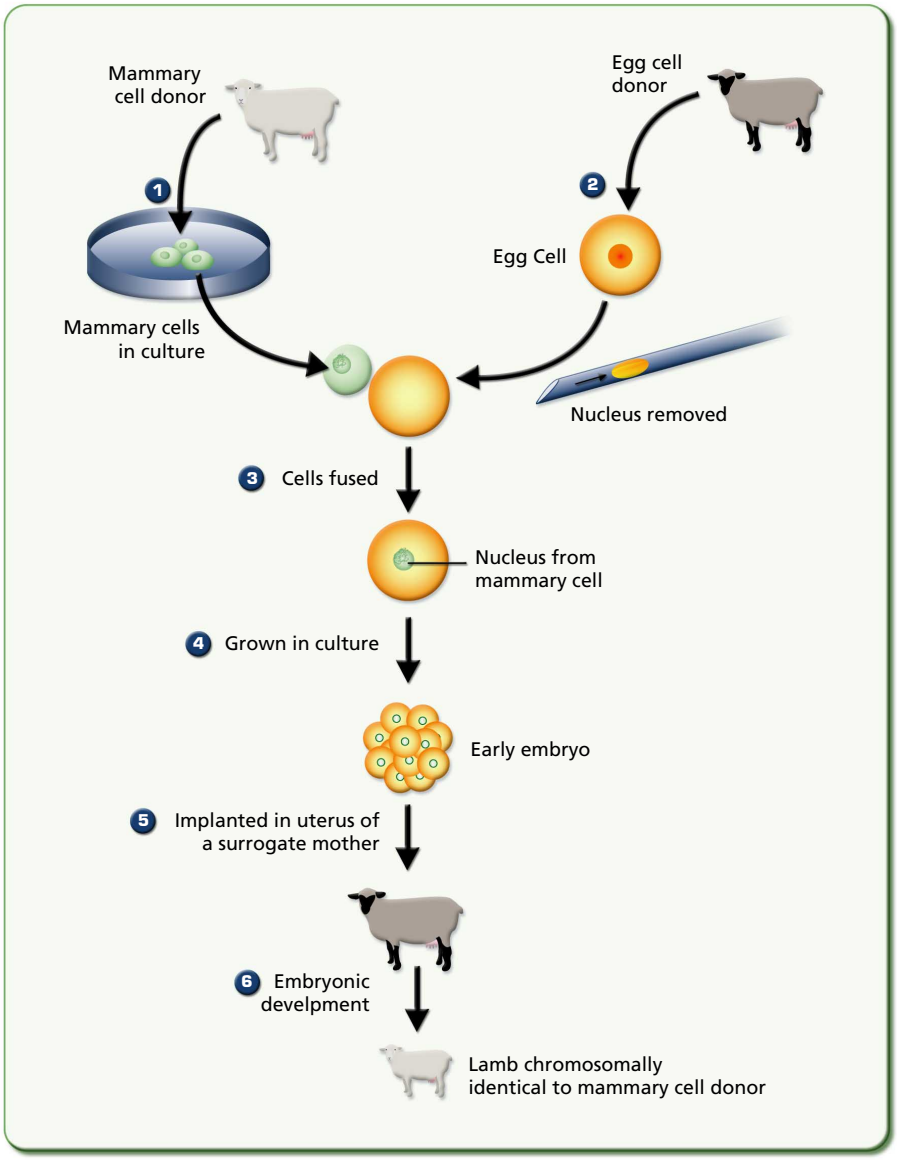


Figure 5. A donor cell is fused with enucleated egg cell by subjecting the two cells to pulses of electricity. The cell replicates in culture, generating an embryo, which is then introduced into the uterus of a female for development.

Nuclear Transfer

Ian Wilmut and his colleagues cloned Dolly using a technique called nuclear transfer[1]. In this technique, the nucleus of a recipient egg is removed to make way for the genetic material of the donor. (**Fig. 5**) The donor cell is fused with the enucleated egg cell by subjecting the two cells to pulses of electricity. Earlier studies had suggested that donor nuclei from early embryos were more likely develop properly. The use of an adult cell for the donor nucleus was unique in Dolly's

case. Although most differentiated animal cells contain all the genes for making an entire organism, nuclei change as cells differentiate. To dedifferentiate the udder cells used for nuclear transfer they cultured the cells in a nutrient-poor medium. This caused the cell cycle to stop in the GO phase. After fusion, 277 embryos were grown in culture for six days before implanting them in thirteen surrogate mothers. Only one of the embryos completed normal development.

Cloning by nuclear transfer depends on the availability of donor cells with the appropriate genetic information. Somatic cells such as fibroblasts, ovarian cells, muscle cells, and mammary epithelia are grown in cell culture and genetically modified by fusion with the enucleated egg. Commonly, DNA is transferred to the cells using viruses.

Microinjection and Other Techniques

Another technique for cloning animals is microinjection. In this technique, a gene construct is characterized in culture and an adequate quantity of the desired DNA is obtained. The DNA is injected into fertilized ova before the first cell division occurs. This increases the probability that all of the cells of the organisms will harbor the gene. The injection is done soon after fertilization, before the male and female pronuclei have fused. A very thin pipette or needle injects the DNA into the large male pronucleus. (**Fig. 6**) Surrogate mothers are made pseudo-pregnant with hormones and implanted with the injected eggs. After birth, tissue samples of the young are assessed for the presence of the desired gene. DNA from germ line cells is given special attention. If the novel gene is present in these cells, the animal can be used as a founder for breeding.

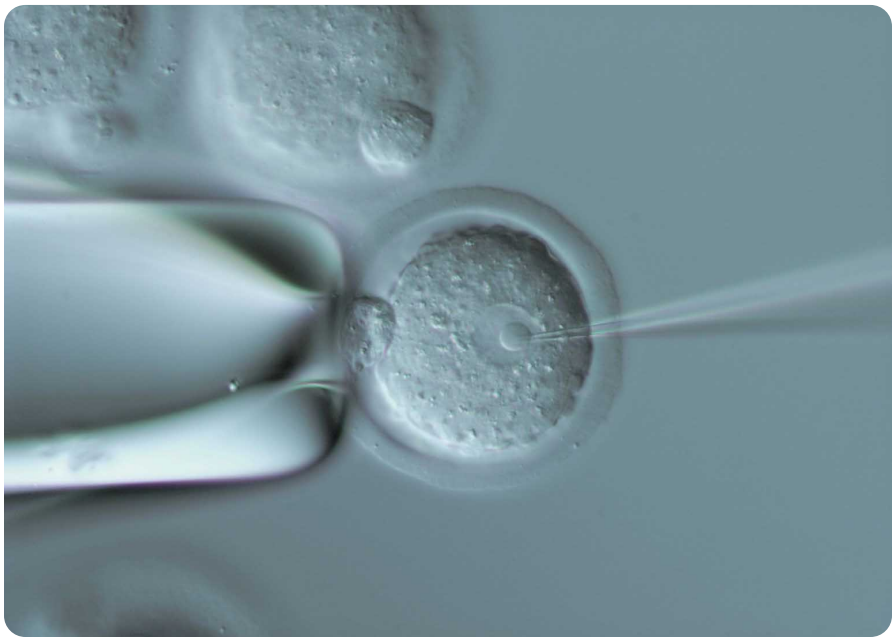


Figure 6. Microinjection.

Genetic constructs that include regulatory regions targeting gene expression to specific tissues are necessary if the gene product is to be harvested readily. For example, GTC Biotherapeutics uses the betacasein promoter to ensure that antithrombin III is secreted in goat milk. Common biochemical procedures, such as filtration and chromatography, are then used to isolate the AT-III from the milk.

Scientists often use **southern blots** to evaluate DNA extracts from tissue samples. (**Fig. 7**) Southern blotting is a type of nucleic acid hybridization test in which single-stranded DNA from two sources interact. Strands with similar nucleic acid sequences will anneal by base pairing (A with T, and G with C) to form double-stranded molecules. One of the single-stranded DNA molecules is a unique portion of the gene of interest, and is radiolabeled so it can be detected on photographic film (the probe). Southern blotting allows the detection of fragments of genomic DNA, which anneal to the radiolabeled probe. The fragments are generated using restriction enzymes and separated in a gel by electrophoresis. The size of a given fragment relates to the distance it migrates on electrophoresis. The fragments are denatured to single strands, transferred to a special filter paper that is immersed in a solution containing the probe, and then rinsed. If the probe has annealed it will expose the photographic film, resulting in a band.

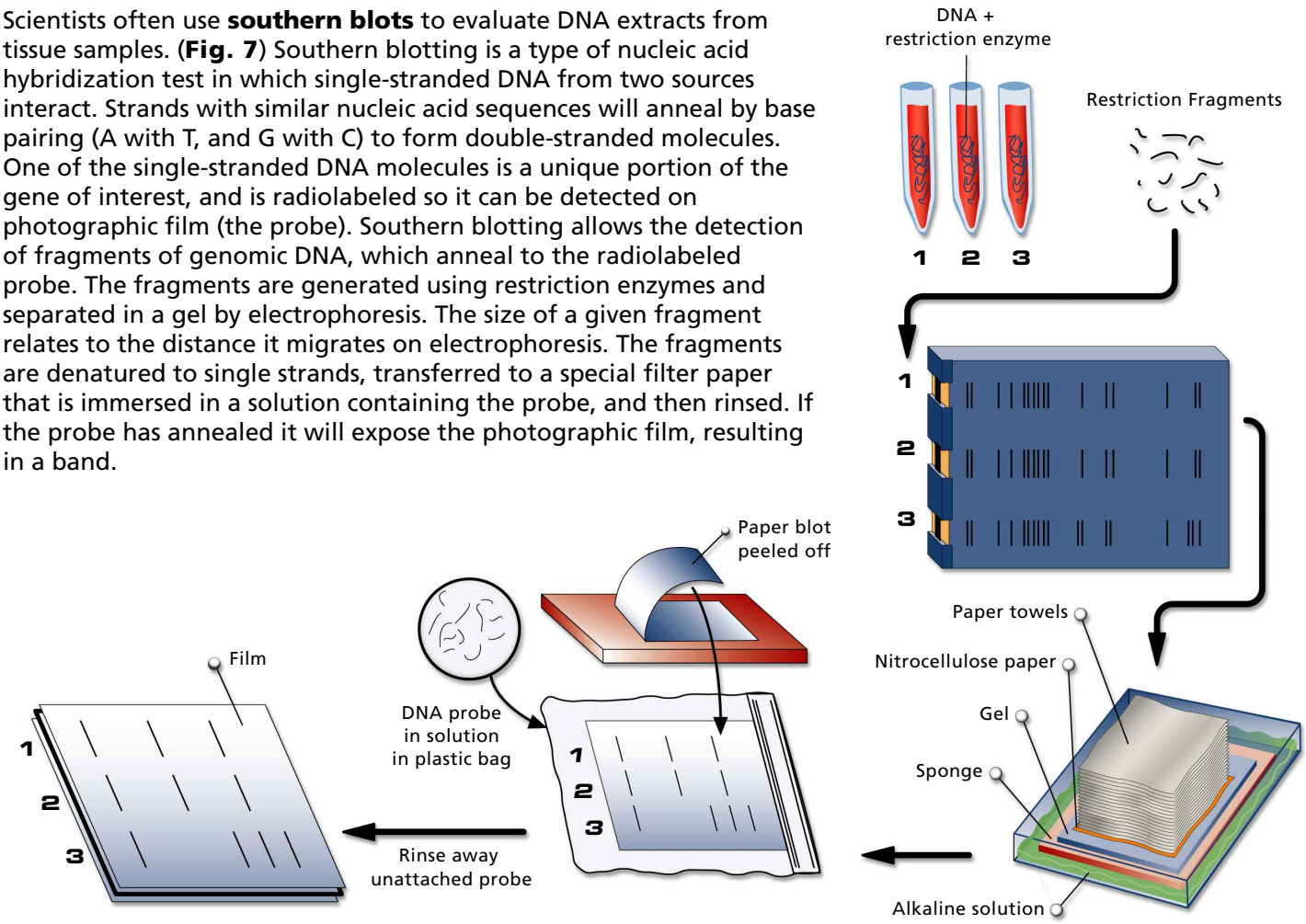


Figure 7. DNA fragments are generated using restriction enzymes. The fragments are separated in a gel by the application of an electric charge. The fragments are then blotted onto a piece of nitrocellulose paper, where they retain their same pattern of separation but are denatured to become single-stranded DNA. A unique single-stranded portion of the gene of interest (the probe) is radioactively labeled and allowed to anneal with the blotted paper. When exposed to a sheet of photographic film, any DNA fragments that annealed with the labeled probe are identified.

Challenges

Even beyond the controversies involving human cloning, there are risks and ethical dilemmas surrounding the use of transgenic and cloned animals.

One risk from cloning animals is a loss of genetic diversity in livestock. This could result in increased susceptibility to disease or other environmental challenges. Some of this risk might be avoided, according to the Roslin Institute, by systems that limit the number of clones produced by breeders and restricting the number of clones sold to any given farmer.

The overexpression or deletion of certain genes must also be evaluated from an animal welfare perspective. The secretion of proteins in the milk of transgenic goats seems to have no ill effects. However, pigs that harbor foreign genes have exhibited many problems including lameness, lethargy, thickened skin, kidney dysfunction, inflamed joints, peptic ulcers, pericarditis, severe osteoarthritis, and a propensity toward pneumonia.

The safety of cloning techniques has been questioned by a number of researchers. Rudolf Jaenisch (MIT) published a study in September 2002 comparing 10,000 genes from placentas and livers of newborn cloned mice with those from normal mice; at least four percent were functioning incorrectly. Cloned mice have exhibited developmental

abnormalities, obesity, pneumonia, liver failure, and premature death. Dolly exhibited arthritis at an unusually young age and was put to sleep at age six, about half the life expectancy of sheep in captivity.

An additional concern about the use of transgenic animal products, including transplanted organs, is the risk of human exposure to animal pathogens. At least 150 pathogens are known to infect both humans and some other animal. In 1997 the isolation of two retroviruses from pigs that could infect human tissue culture cells was reported. These so called PERVs (porcine endogenous retroviruses) are of special concern to those considering the use of porcine tissue for transplants, especially because some retroviruses have been associated with cancer.

Addressing the Controversies

Decisions made regarding the use of genetically modified organisms will impact the environment, and force a reexamination of consumer safety and animal welfare issues. Do the benefits provided by transgenic organisms outweigh the risks? Are those making decisions influenced too heavily by the profit motive? How can opportunities for competing approaches be ensured?

Certainly the production of genetically altered organisms is a profit-making business. In 1980, individuals and companies realizing this sought protection of their intellectual property and turned to the courts. That year the U.S. Supreme Court delivered a landmark decision stating that living organisms are patentable; in 1988 a patent was issued for the genetically altered "Harvard mouse."

In late 2001 seventy-seven scientists and teachers from sixteen countries, concerned with how environmental protection decisions are made, issued the Lowell Statement on Science and Precaution. Their "Precautionary Principle" recommends using the safest approaches to meeting society's needs, placing responsibility for finding the safest alternatives in the hands of those originating potentially dangerous activities, use of independent review, and participation of those who may be affected by a policy choice. These guidelines might well be extended beyond environmental policy.

Governmental bodies often play the role of reviewer when it comes to safety, particularly of foods. Various governments and organizations have begun generating guidelines and recommendations regarding foods derived from transgenic organisms. For example, the Food and Agricultural Organization of the United Nations along with the World Health Organization organized a series of scientific consultations to provide their member nations with recommendations. In a January 2001 report the consultation agreed that "the safety assessment of foods derived from biotechnology requires an integrated and stepwise, case-by-case approach."[2]

Can the population at large — by consumer and political choice — influence the use of genetically modified organisms? In November 2002 Oregon was the first state in the U.S. to put labeling of genetically modified foods on the ballot. Proponents of labeling spent about 200,000 dollars to convince voters. Opponents, with funding from large agribusinesses, spent 5.5 million dollars to kill the idea. Voters were convinced that labeling would significantly increase food costs and rejected the measure.

References

1) Wilmut I., A. E. Schnieke, J. McWhir, A. J. Kind, and K. H. S. Canbell. 1997. Viable offspring derived from fetal and adult mammalian cells. *Nature* 385:810–12.

2) Food and Agriculture Organization of the United Nations (FAO). 2001. *Evaluation of allergenicity of genetically modified foods.* Report of a joint FAO/WHO expert consultation on allergenicity of food derived from biotechnology 22–25 Jan 2001. Rome, Italy

Further Reading

Books

Crotty, S. 2001. *Ahead of the curve: David Baltimore's life in science*. Berkeley, CA: University of California Press.
A biography of one of the major figures in molecular genetics and recombinant DNA technology.

Nelson, G. C., ed. 2001. *Genetically modified organisms in agriculture — Economics and politics.* New York: Academic Press.
This book presents opinions regarding the use of genetically modified crops and uses citations from the scientific literature as a foundation for those opinions.

Articles

Food and Agriculture Organization of the United Nations (FAO). 2001. *Evaluation of allergenicity of genetically modified foods.* Report of a joint FAO/WHO expert consultation on allergenicity of food derived from biotechnology 22–25 Jan 2001. Rome, Italy
A report and recommendations from a group of experts who met under the auspices of the United Nations.

Lowell Statement on Science and the Precautionary Principle. 2001. *Statement from the international summit on science and the precautionary principle*. Hosted by the Lowell Center for Sustainable Production, University of Massachusetts, Lowell; 20–22 September 2001. http://www.uml.edu/centers/lcsp/precaution.
The precautionary principle developed by this group is provided in full at this Web site.

Moffat, A. S. 1998. Toting up the early harvest of transgenic plants. *Science* 282:2176–78.
A brief review of recent advances in transgenic agriculture.

Wolfenbarger, L. L., and P. R. Phifer. 2000. The ecological risks and benefits of genetically engineered plants. *Science* 292:637–38.
A review of the ecological considerations inherent to the development of genetically engineered crops.

Ye, X., A. A. Babili, A. Kloti, J. Zhang, P. Lucca, P. Beyer, and I. Potrykus. 2000. Engineering the provitamin A beta-carotene biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* 287:303–5.
The scientific paper that presents the development of "golden rice."

Glossary

Bt. The bacterium *Bacillus thuringiensis*; also refers to the crystalline insecticidal protein produced by the bacterium. Bt crops, such as Bt-corn, are transgenic plants that express the insecticidal protein.

Clone. Two or more genetically identical progeny. Clones can be of genes, cells, or whole organisms.

Cloning vector. A carrier of DNA that can replicate; usually a plasmid, bacteriophage, or eukaryotic virus.

Electroporation. Use of electric shock to make cell membranes temporarily more permeable to molecules such as DNA.

Exon. The sequence of a gene that encodes a protein. Exons may be separated by introns.

Gene gun. A device that delivers DNA to cells by microprojectile bombardment.

Intron. The DNA sequence within a gene that interrupts the protein-coding sequence of a gene. It is transcribed into RNA but it is removed before the RNA is translated into protein.

Marker gene. A gene, such as one that encodes antibiotic resistance, that allows genetically modified cells to be readily selected.

mRNA splicing. In eukaryotic cells, the process of excising introns from a primary RNA transcript and joining together exons to form a final mRNA molecule.

Plasmid. A small, circular, self-replicating, extrachromosomal piece of DNA. Many artificially constructed plasmids are used as cloning vectors.

Recombinant DNA. DNA that contains information from two or more different species of organisms.

Restriction enzymes. Enzymes that cut DNA at specific sequences; also known as "restriction endonucleases."

Reverse transcriptase. An enzyme derived from a retrovirus, which uses single-stranded RNA as a template for the production of double-stranded DNA.

Southern blot. A technique for transferring DNA fragments separated by electrophoresis to a filter paper sheet. The fragments are then probed with a labeled, complementary nucleic acid to help determine their positions.

Totipotent. Cells that can replicate to form any part of a complete organism.

Transgenic organism. An organism that contains hereditary information from two different species of organisms.